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STRUCTURE OF THE CAROTID BODY TUMOR

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The carotid body tumor, termed "paraganglioma" by Herxheimer,[1] "nonchromaffin paraganglioma" by Lattes and Waltner[2] (based on Penitschka's[3] and Watzka's[4] conceptions of the normal carotid body), and "chemodectoma" by Mulligan,[5] is a relatively rare neoplasm. It is known to remain stationary during a period of several years without causing clinical symptoms of importance and is often confused clinically with a caseous lymph node or an aneurysm of the carotid artery. In the majority of cases the correct diagnosis is arrived at only through histologic examination. Until quite recently the surgical removal of the tumor caused a high mortality since the common carotid artery had to be ligated, but advances in the operative technique have improved the prognosis considerably.[6] In spite of the apparent minor clinicopathologic importance attributed to this tumor, publications concerned with it are abundant.

An excellent description of the microscopic structure of carotid body tumor is that of Dietrich and Siegmund[7]; we are also indebted to them for a very complete review of the literature up to 1926. The extensive work of LeCompte[8] was published in 1951. Other previous reviews are those of Klose,[9] Schmidt,[10] Brines and Jennings,[11] Herzog,[12] and Masson.[13]

MATERIAL AND METHOD

We have had the opportunity to study 6 cases of carotid body tumor. All were fixed in 10 per cent formaldehyde, and fragments of 2 of them were also fixed in Cajal's ammonium bromide-formalin. Sections were prepared by both paraffin and

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frozen section methods and were stained by hematoxylin and eosin and by Gallego's trichrome stain for connective tissue, as well as by variants of this stain for elastic fibers, mast cells and cytoplasmic granules. The silver impregnation methods of Gros were employed for staining peripheral nerve fibers, and the multiple methods devised by del Río Hortega [14] were used to demonstrate reticular fibers, nerve fibrils and neuroglial fibers.

Observations

The silver impregnation methods are especially valuable in morphologic research because, when properly used, they display the unique preferential properties of staining either a single structure or several structures comprising the tissue under consideration. In order to attain such flexible specificity it is enough to vary, occasionally slightly, the time of fixation, the nature of the fixative, the formula of the impregnating solution, the temperature or the time of impregnation, and the physical conditions of the reduction. If these variables are intelligently utilized, outstanding results can be achieved with silver impregnation.

The impregnation methods used in this study have enabled us to demonstrate for the first time the presence of cells with argentaffin granules in carotid body tumors. These are morphologically similar to the Kultschitzky [15] cells in the gastrointestinal tract. We have also been able to make original observations concerning the structure of the chief cells, which assume a neuronoid appearance similar to that of other receptor cells, and the presence of numerous nerve fibers with synaptic connections frequently associated with the chief cells.

Argentaffin Cells

In various areas in 2 of the 6 tumors examined, we observed variable numbers of cells with intracytoplasmic argentaffin granules. In both cases, specimens were fixed in 10 per cent aqueous formalin solution. In the identification of these granules the criteria suggested by Masson [16] and accepted by Lillie and Glenner [17] were followed; the granules reduced silver salts in ammonium solutions without the necessity of using an extraneous reducing reagent.

When argentaffin cells were numerous in the carotid body tumor, they tended to be distributed at the margins of the tumor lobules. Although they appeared to be adjacent to blood vessels, they apparently were not actually in the vessel walls but lay rather in strands of connective tissue supporting the vessels and separating the neoplastic lobules. Argentaffin cells were observed among the chief cells only in those areas where the tumor had completely lost its lobular arrangement. The argentaffin cells with peripheral lobular distribution were spread out in a remarkably uniform manner (Fig. 1), and from the evenness of this pattern there

was a distinct impression that these constituted an important histologic component of the neoplasm. The cells appeared in significant numbers and with clear design; they were neither sparse nor irregularly distributed.

The nucleus of the argentaffin cell was always single and occupied a central position. The framework of chromatin had no outstanding or peculiar features. The granules were numerous and quite tightly packed. They appeared round or slightly elongated and when impregnated with silver, frequently hid the nucleus under a thick black mantle. The size of the argentaffin cells was relatively large and the granular cytoplasm abundant. With silver stains they occupied almost twice the space taken up by the nongranular chief cells. Configuration varied; peripherally, the contours were rounded, but elsewhere the cells were spheroid, fusiform or flattened and, occasionally, exhibited ameboid prolongations. Whatever its shape, the cytoplasm was always densely filled with argentaffin granules (Fig. 2).

Chief Cells

During the past 6 years there has been a tendency to distinguish two classes of chief cells in the normal carotid body and in the carotid body tumor. These cells have been designated "light" and "dark," according to the intensity of staining with aniline dyes,[18–20] or "argentophil" and "nonargentophil," according to their affinity for silver with methods used for staining neuronal elements.[21] The latter classification is based on the concepts of Honjin[22] concerning nerve cells which form the Meissner and Auerbach plexuses in the gastrointestinal tract. Others, however, have considered these staining differences to indicate artifacts of fixation or functional variations.

With del Río Hortega's ammonium-silver carbonate method for glia cells, the parenchyma of the carotid body tumor was observed to be made up of cells, all of the same type, with thick, elongated processes (Fig. 3). The majority of these chief cells were pyriform, and their sole prolongations neither subdivided nor gave origin to smaller branches. Generally speaking, one could not see the full length of the prolongation in one slide since it was quite long as well as tortuous; tracing its course required the use of serial sections. In most of the cells the perikaryon was reduced to a narrow band of cytoplasm somewhat thickened and strengthened at its site of origin. The cytoplasm was homogeneous, transparent, uniformly and moderately argentophilic, and lacked the vacuoles or granules which have always been of such concern to students of the normal carotid body. The prolongation came to an end at a variable distance in a thick, bulbous mass or club. This was often found

adjacent to other similar masses and close to the vascular sinusoids (Figs. 4 and 5).

The overall histologic appearance in the carotid body tumor, then, is that of a rather loose network formed by the irregularly tangled processes which extend from the chief cells and form a complex and heterogeneous pattern (Fig. 3). The significance of this is difficult to interpret. Using Gros's method, which stains only some of the cells, including their prolongations, one gathers the impression that there is not a dominant pattern either for the cell bodies or for their prolongations (Fig. 4). Nonetheless, with the del Río Hortega method which stains all the cells and their processes with homogeneous intensity, multiple processes are observed to form dense bundles close to the blood vessels and end up forming clusters of clubs in close proximity to the endothelial wall of sinusoidal capillaries. With the Gros stain some of these bundles are noted to be oriented perpendicularly to the course of the blood vessels which limit each tumor lobule. This orientation is the opposite of that followed by the nerve fibers which are frequently attached to blood vessel adventitia, and follows very precisely the course of the capillaries, as detailed in the following paragraphs.

The terminal clubs of the chief cell prolongations in carotid body tumors are pleomorphic. In some areas there are many long processes, crossing in different directions and terminating near the endothelium lining the sinusoidal spaces (Fig. 4). It is important to note that the clubs do not, in any way, form synaptic connections but rather lie free in intercellular spaces. In other areas multiple terminal clubs are lined up in such a manner as to resemble a vault which is curved in the course of the adjacent capillary (Fig. 5).

Nerve Fibers

The capsule and many of the thick connective tissue trabeculae penetrating into the deepest zones of the tumor frequently contain bundles of nerve fibers (Fig. 6). Each fiber follows a gently wandering course, and each shows variation in its thickness. Altogether the fibers show a definite pattern of association. All of these factors are characteristic of normal nerve bundles.

The nerve bundles, on entering the carotid body tumor, are frequently related to the large sinusoidal spaces and can be distinguished running in contiguity with the endothelial lining over wide zones (Fig. 7). This intimate association of nerves and blood vessels is also seen in the normal carotid body. Nerve endings present in the carotid body tumor assume 3 main forms: (a) *"boutons terminaux,"* which cluster together but are independent of one another; these originate from arched interstitial

nerve fibrils which exhibit typical intercalated nodule formation (Fig. 8); (b) end plates which have origin in coarse nerve fibers (Fig. 9); and (c) multiple end fibrils which form typical pericellular baskets (Fig. 10).

Discussion

In a review of the literature concerning the normal carotid body and carotid body tumors, we have encountered but one mention of cells containing argentaffin granules. Feyrter demonstrated abundant argentaffin cells in the normal human carotid body with the Bielschowsky-Gros technique.[23] Our colleague, Dr. A. Villasana,[24] using Fontana's method, has also been successful in demonstrating argentaffin granules in cells of the normal human carotid body. We have had the opportunity of examining the sections kindly provided for us by Dr. Villasana, and we are convinced that the aforementioned cells are evident in the normal organ.

The argentaffin cells in the carotid body tumors appear to be morphologically similar to those in the gastrointestinal tract as well as those recently described by Grasso[25] in the urethra and prostate gland. Since the argentaffin granules in Kultschitzky cells are thought to be involved in serotonin production,[26,27] it is possible that those in the carotid body tumors may also secrete serotonin (5-hydroxytryptamine), a substance which, according to Erspamer and Asero,[28] Lembeck,[29] and Page,[30] is a vasopressor of great biologic importance.

The fact that argentaffin cells were not found in all of the tumors which we analyzed and that, when found, they only appeared with a patchy distribution even in the tumors containing a great number of them, does not, in our opinion, make their discovery any less important. Even in the normal intestine, they are characterized by great variations in distribution.[31–33] This is also the case in argentaffin cells in the prostate gland,[25] and in the normal human carotid body.[24] On the other hand, the distribution of serotonin in human tissues indicates that the intestinal mucosa is not the sole site of its production.[30,34]

Histologists have demonstrated an abundant afferent innervation originating in the chief cells of the normal carotid body,[35–37] and physiologists have shown that the carotid body acts as a chemoreceptor.[38–41] There is therefore no reason to doubt that the chief cells in carotid body tumors are specialized receptor elements. With the foregoing in mind, we feel that the term "chemodectoma" suggested by Mulligan[5] is a very suitable designation for this lesion. Although it is generally accepted that the carotid body is a "paraganglion," it seems more accurate, in the light of recent advances, to consider it as a receptor. If we consider the work on the normal carotid body by Martínez,[42] Willis and Tange,[20] and Ross,[43] and the studies of Willis and Birrel[21] as well as the present

investigation on the carotid body tumor, the conclusion may be reached that the chief cells are "argentophil" in technical circumstances similar to those which prevail when some neurons show the same property. Argentophilia is also a common quality in many varieties of neuroglia and in certain specialized receptors such as the rods and cones of the retina, olfactory cells, and taste buds.

The differences in form, structure, and staining which have been observed in normal and neoplastic cells in the carotid body when the silver impregnation methods of Bielschowsky or of Gros were used, seem to us to be the result of technical artifacts and to have no real importance as far as the possibility of different cellular varieties is concerned. The techniques of del Río Hortega indicate that all of the chief cells in carotid body tumors are of the same type and constitute a single morphologic entity.

Finally, we believe that it is significant and important that all of the carotid body tumors we have studied contained an abundant innervation. Since there is no anatomic or functional reason to suspect that the innervation of the carotid body tumor has essential differences from that in the non-neoplastic organ, we can assume that the carotid body tumor fulfills functions which are similar if not identical to those of the normal organ. Nerve fibers have been seen in carotid body tumors by Chase[44] and Gordon-Taylor,[45] but they have not always been closely associated with the neoplastic parenchyma and have been seen only in very small numbers. The majority of those who have written on this subject have not mentioned the existence of nerve fibers, or have considered them to be inconspicuous and unrelated to the tumor itself.[9,46,47]

Summary

The silver impregnation methods of del Río Hortega and Gros demonstrate the morphologic properties of receptor cells in the chief cells of carotid body tumors. Stained with silver, these appear as pyriform cells with single, long, thick processes exhibiting clublike endings. The clubbed ends do not exhibit synaptic connections, at least with the staining methods employed. The clubs are generally grouped around blood vessels although some appear as isolated elements among other chief cells. The prolongations are so long and tortuous that their origin and termination do not generally appear in the same plane. They pass from one tumor lobule to another, occasionally forming thick bundles which cross blood vessels and end at various distances from their points of origin.

In addition to the chief cells, some carotid body tumors contain argentaffin cells which are morphologically similar to the Kultschitzky

cells of the gastrointestinal tract. When found, these are very abundant and exhibit an organoid distribution among the delicate connective tissue strands and capillary sinusoids which form the boundaries of tumor lobules.

In the absence of fibrosis and atrophy, all of the neoplastic lobules are innervated by a prominent framework of nerve fibrils which have a normal appearance and are equipped with *"boutons terminaux,"* end plates, and terminal baskets synaptically related to the parenchymal cells.

References

1. Herxheimer, G. Ueber Tumoren des Nebennierenmarkes, insbesondere das Neuroblastoma sympathicum. *Beitr. path. Anat.*, 1913, 57, 112–117.
2. Lattes, R., and Waltner, J. G. Nonchromaffin paraganglioma of the middle ear (carotid-body-like tumor; glomus jugulare tumor). *Cancer*, 1949, 2, 447–468.
3. Penitschka, W. Paraganglion aorticum supracardiale. *Ztschr. mikr.-anat. Forsch.*, 1931, 24, 24–37.
4. Watzka, M. Über die Entwicklung des Paraganglion caroticum der Säugetiere. *Ztschr. Anat.*, 1937, 108, 61–73.
5. Mulligan, R. M. Chemodectoma in the dog. (Abstract) *Am. J. Path.*, 1950, 26, 680–681.
6. Monro, R. S. The natural history of carotid body tumours and their diagnosis and treatment, with report of 5 cases. *Brit. J. Surg.*, 1950, 37, 445–453.
7. Dietrich, A., and Siegmund, H. Die Nebenniere und das chromaffine System (Paraganglien, Karotisdrüse, Steissdrüse). XI. Chromaffines System (Paraganglien, Karotisdrüse, Steissdrüse). (b) Die Karotisdrüse. In: Handbuch der speziellen pathologischen Anatomie und Histologie. Henke, F., and Lubarsch, O. (eds.) J. Springer, Berlin, 1926, Vol. 8, Drüsen mit innerer Sekretion, pp. 1060–1071.
8. Le Compte, P. M. Tumors of the Carotid Body and Related Structures (Chemoreceptor System). Atlas of Tumor Pathology, Section IV, Fascicle 16, 40 pp. Subcommittee on Oncology of the Committee on Pathology of the National Research Council. Armed Forces Institute of Pathology, Washington, D.C., 1951.
9. Klose, H. Beitrage zur Chirurgie der sogennante Carotisdrüse. *Arch. klin. Chir.*, 1922, 121, 689–724.
10. Schmidt, P. Der jetztige Stand unserer Kenntnisse über die Glandula carotica. Inaugural dissertation, Marburg, 1922.
11. Brines, O. A., and Jennings, E. R. Paragangliomas; review of subject and report of five original cases. *Am. J. Path.*, 1948, 24, 1167–1197.
12. Herzog, E. Histopathologie des vegetativen Nervensystems. In: Handbuch der speziellen pathologischen Anatomie und Histologie. Henke, F., and Lubarsch, O. (eds.). J. Springer, Berlin, 1954, Vol. 13, Nervensystem; part 5, Erkrankungen des peripheren Nervensystems; Erkrankungen des vegetativen Nervensystems, pp. 357–542.
13. Masson, P. Tumeurs Humaines. Histologie—Diagnostics et Techniques. Maloine, Paris, 1956, ed. 2, 1216 pp.

14. DEL RÍO HORTEGA, P. El método del carbonato argéntico. Revisión general de sus técnicas y aplicaciones en histología normal y patológica. *Arch. histol. norm. y pat.*, 1942, **1**, 165–205; 329–361; and 1943–1945, **2**, 231–244; 577–604.

15. KULTSCHITSKY, N. Zur Frage über den Bau des Darmkanals. *Arch. mikr. Anat.*, 1897, **49**, 7–35.

16. MASSON, P. La glande endocrine de l'intestine chez l'homme. *Compt. rend. Acad. de sc.*, 1914, **158**, 59–61.

17. LILLIE, R. D., and GLENNER, G. G. Histochemical reactions in carcinoid tumors of the human gastrointestinal tract. *Am. J. Path.*, 1960, **36**, 623–651.

18. DE KOCH, L. L. The intraglomerular tissues of the carotid body. *Acta anat.*, 1954, **21**, 101–116.

19. GARNER, C. M., and DUNCAN, D. Observations on the fine structure of the carotid body. *Anat. Rec.* 1958, **130**, 691–709.

20. WILLIS, A. G., and TANGE, J. D. The argentophil cells of the human carotid body. *Am. J. Anat.*, 1959, **105**, 141–163.

21. WILLIS, A. G., and BIRRELL, J. H. W. The structure of a carotid body tumor. *Acta anat.*, 1955, **25**, 220–265.

22. HONJIN, R. The distribution and morphology of argentophil and argentophobe nerve cells in the myenteric plexus of the digestive tube. (Abstract) *Anat. Rec.*, 1960, **136**, 212.

23. FEYRTER, F. Zur Normung der Silberimprägnation neuraler and nichtneuraler Gewebe. *Virchows Arch. path. Anat.*, 1951, **320**, 551–563.

24. VILLASANA, A. Personal communication.

25. GRASSO, R. Sobre las células argentafines de la uretra y de la glándula prostática. *Arch. histol. norm. y pat.*, 1954, **5**, 227–270.

26. BARTER, R., and PEARSE, A. G. E. Mammalian enterochromaffin cells as the source of serotonin (5-hydroxytryptamine). *J. Path. & Bact.*, 1955, **69**, 25–31.

27. PEARSE, A. G. E. A contribution to the chemistry of mammalian enterochromaffin cells. *Riv. istochim. norm. e pat.*, 1956, **12**, 103–110.

28. ERSPAMER, V., and ASERO, B. Identification of enteramine, the specific hormone of enterochromaffin cell system, as 5-hydroxytryptamine. *Nature, London*, 1952, **169**, 800–801.

29. LEMBECK, F. 5-Hydroxytryptamine in a carcinoid tumour. (Letter to the editor.) *Nature, London*, 1953, **172**, 910–911.

30. PAGE, I. H. Serotonin (5-hydroxytryptamine). *Physiol. Rev.*, 1954, **34**, 563–588.

31. TEHVER, J. Ueber die enterochromaffinen Zellen der Haussäugetiere. *Ztschr. mikr.-anat. Forsch.*, 1930, **21**, 462–496.

32. SCHUMANN, G. Experimentelle Untersuchung über die basalgekörnten Zellen im Darmepithel des Meerschweinchens. *Ztschr. Zellforsch.*, 1936, **24**, 540–551.

33. HOESCHEN, T. Ueber Menge und Verteilung der basalgekörnten Zellen im Darm des Meerschweinchens. *Ztschr. Zellforsch.*, 1937, **27**, 711–719.

34. WILKINS, R. W. Serotonin, antiserotonins and hypertension. *New England J. Med.*, 1956, **255**, 115–118.

35. DE CASTRO, F. Sur la structure et l'innervation de la glande intercarotidienne (glomus caroticum) de l'homme et des mammifères, et sur un nouveau système d'innervation autonome du nerve glossopharyngien. *Travaux du Lab. de Recherches Biol. de l'Univ. de Madrid*, 1926, **24**, 365–432.

36. BOECKE, J. Nerve Endings, Motor and Sensory. In: Cytology and Cellular Pathology of the Nervous System. PENFIELD, W. (ed). Paul B. Hoeber, Inc., New York, 1932, Vol. 1, pp. 241–315.

37. MEIJLING, H. A. Bau und Innervation von Glomus caroticum and Sinus caroticus. *Acta Neerland. Morph.*, 1938, 1, 193–288.

38. HEYMANS, C.; BOUCKAERT, J. J.; FARBER, S., and HSU, F. J. Influence réflexogène de l'acétylcholine sur les terminaisons nerveuses, chimio-sensitives, du sinus carotidien. *Arch. internat. pharmacodyn.*, 1936, 54, 129–135.

39. VON EULER, U. S.; LILJESTRAND, G., and ZOTTERMAN, Y. Über den Reizmechanismus der Chemorezeptoren im Glomus caroticum. *Acta physiol. scandinav.*, 1941, 1, 383–385.

40. ALVAREZ-BUYLLA, R. Estudio oscilográfico de la actividad eléctrica de los quimio-receptores del seno carotídeo en el perro. *An. Esc. Nac. Cienc. Biol., Mexico*, 1949, 6, 161–173.

41. DALY, I. DE B., and DALY, M. DE B. The effects of stimulation of the carotid body chemoreceptors on pulmonary vascular resistance in the dog. *J. Physiol.*, 1957, 137, 436–446.

42. MARTÍNEZ, M. Contribución a la histología normal y patológica del glomo carotídeo. *Bol. Soc. biol. Concepción (Chile)*, 1939, 13, 107–131.

43. ROSS, L. L. A cytological and histochemical study of the carotid body of the cat. *Anat. Rec.*, 1957, 129, 433–455.

44. CHASE, W. H. Familial and bilateral tumours of the carotid body. *J. Path. & Bact.*, 1933, 36, 1–12.

45. GORDON-TAYLOR, G. On carotid tumours. *Brit. J. Surg.*, 1940, 28, 163–172.

46. EWING, J. Neoplastic Diseases. A Treatise on Tumors. W. B. Saunders Co., Philadelphia, 1940, ed. 4, pp. 384–385.

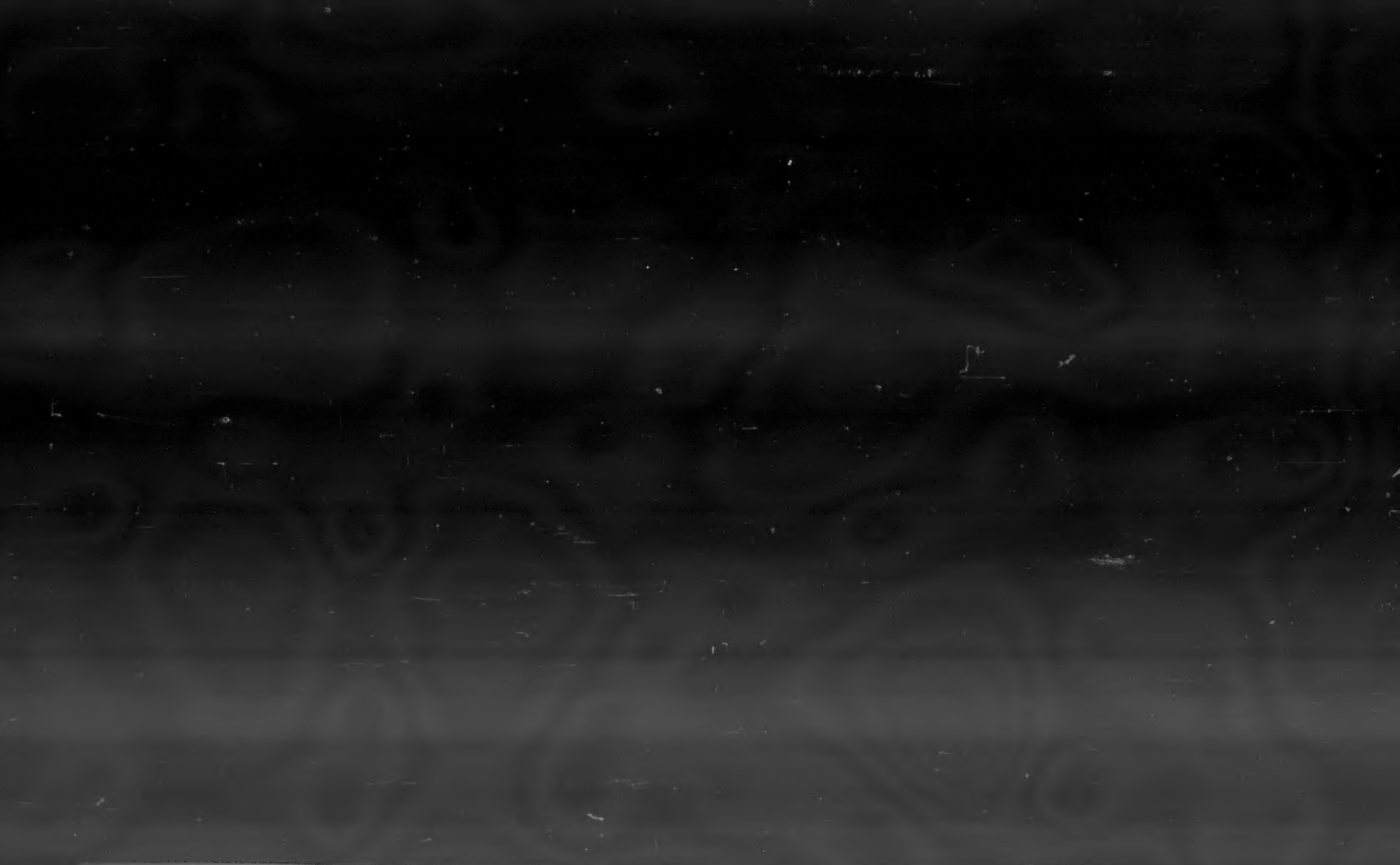
47. EVANS, R. W. Histological Appearances of Tumors. With a Consideration of Their Histogenesis and Certain Aspects of Their Clinical Features and Behaviour. E. & S. Livingstone, Ltd., London, 1956, pp. 75–82.

[*Illustrations follow*]

Legends for Figures

Fig. 1. A panoramic view of the general structure in the carotid body tumor. Argentaffin cells are regularly distributed at the periphery of the lobules. Río Hortega method. × 120.

Fig. 2. Argentaffin cells in a carotid body tumor. Cells containing the intracytoplasmic granulations have a perilobular distribution and are independent of the blood vessels and chief cells. Río Hortega method. × 650.



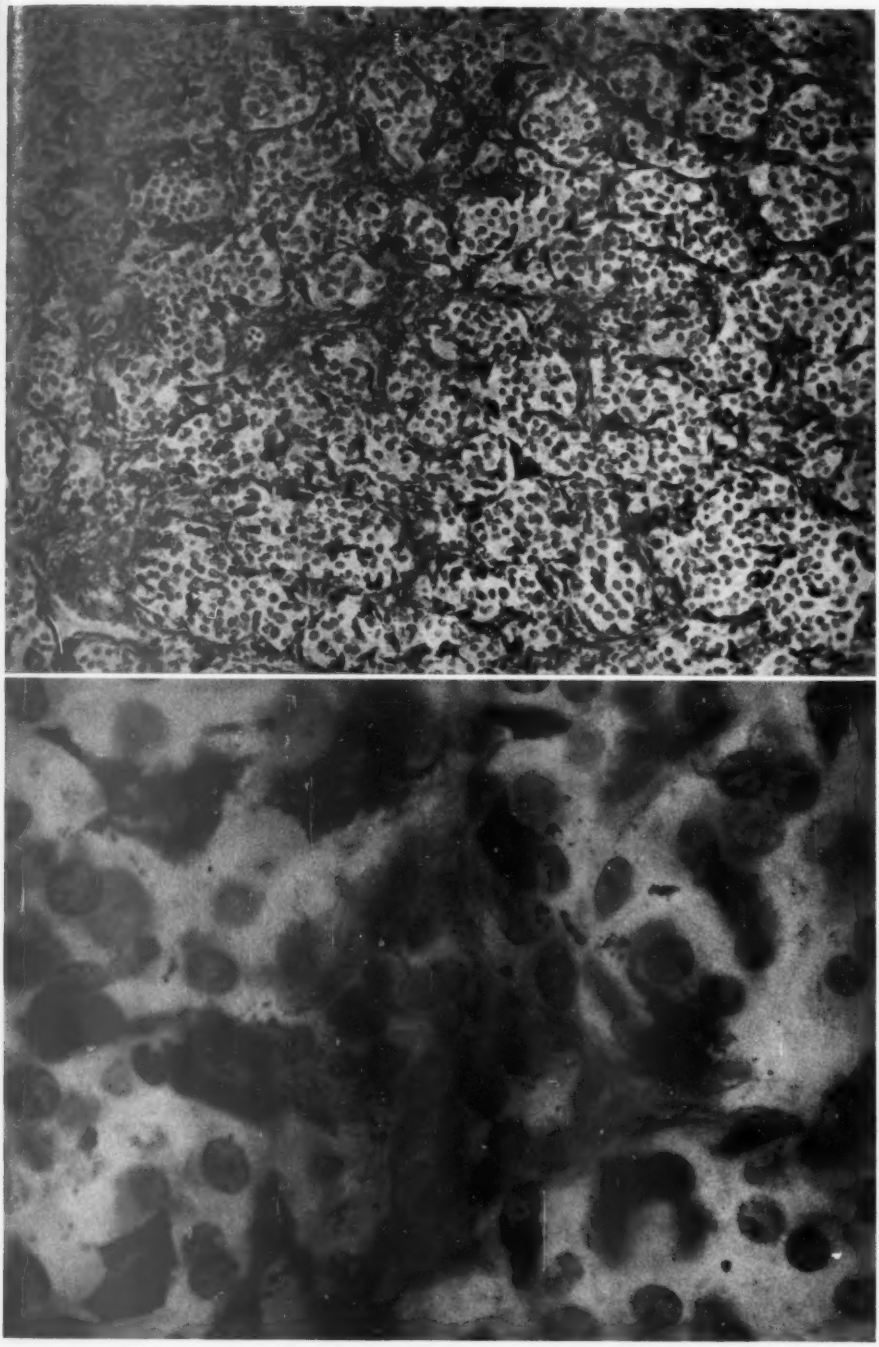


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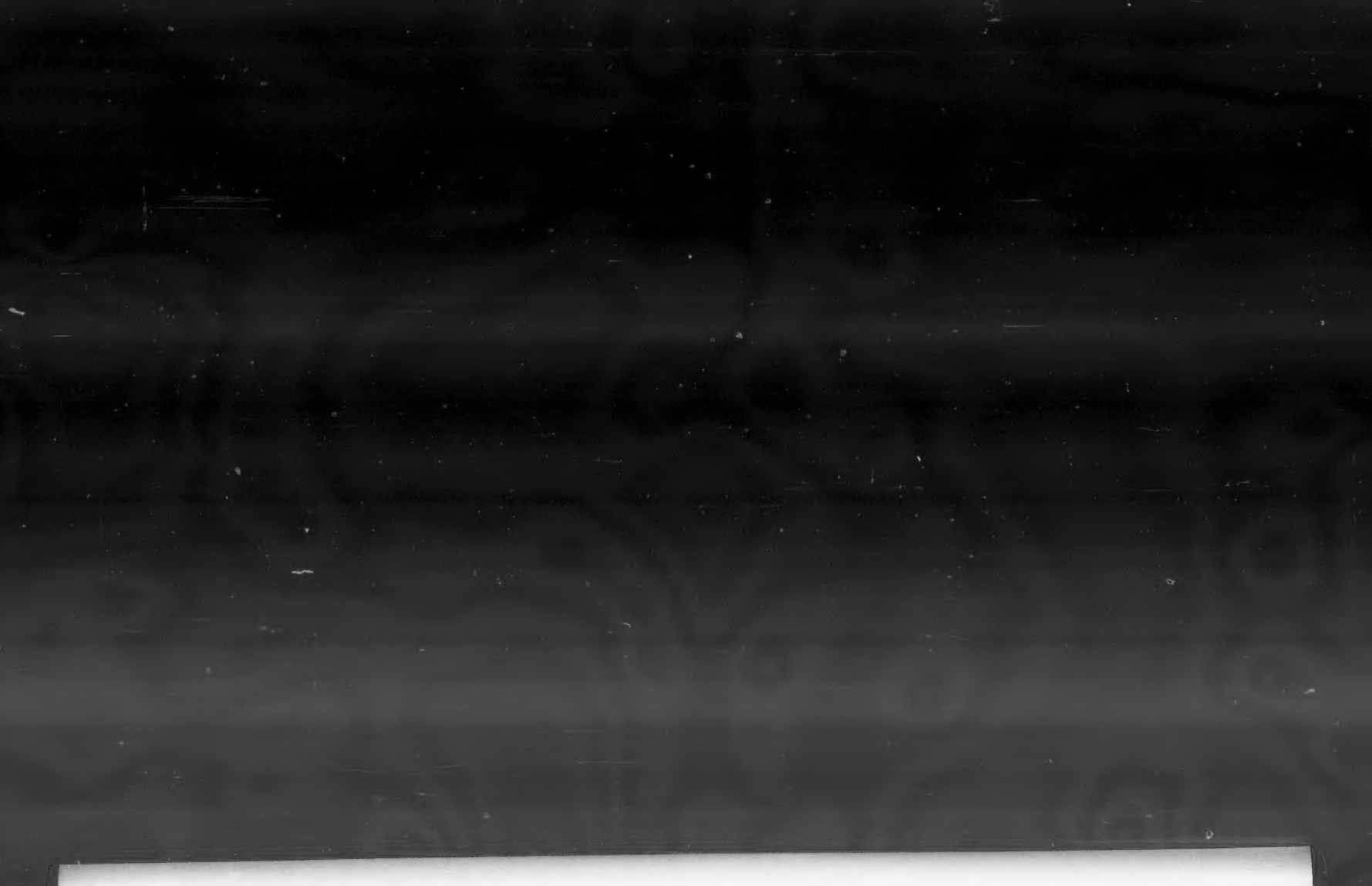
FIG. 3. Chief cells in a carotid body tumor are pyriform and emit a long and broad prolongation. Three of them have been surrounded by a black line in order to make them conspicuous. The chief cells and their prolongations form a loose, irregularly arranged network. Río Hortega method. × 650.

FIG. 4. The chief cell processes in a carotid body tumor are deeply stained. They have no definite orientation, but several of them end in clublike bulges in the neighborhood of sinusoidal walls. Gros method. × 255.



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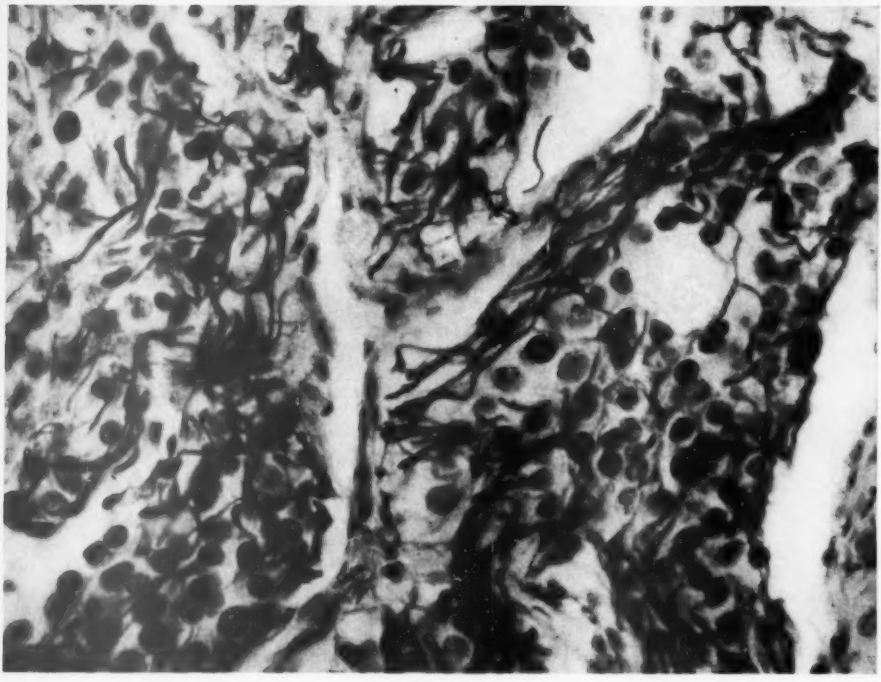

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FIG. 5. Several endings of chief cell processes are gathered beneath the endothelium of a sinusoid in a carotid body tumor. The club-shaped endings in all probability represent active chemoreceptor structures, which transmit impulses to afferent nerve fibers. The latter take origin in synaptic connections with the perikaryon of chief cells. Gros method. × 650.

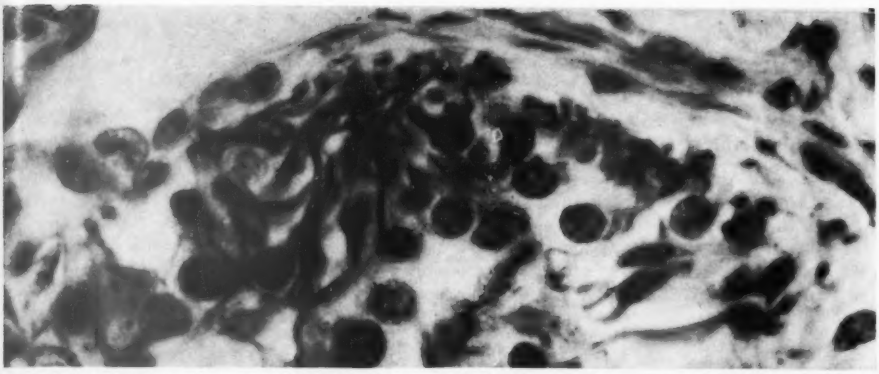
FIG. 6. Nerve fibers with normal morphologic characteristics are abundant in the capsule and broad trabeculations of a carotid body tumor. Río Hortega method. × 255.

FIG. 7. A bundle of nerve fibers is deeply seated within the tumor substance. The bundle runs along the wall of a sinusoid, and isolated fibers are detached from the bundle and penetrate the tumor lobules. Río Hortega method. × 255.

FIG. 8. Some typical "*boutons terminaux*." Gros method. × 650.

FIG. 9. A double ending plate is shown enfolding a chief cell. Gros method. × 650.

FIG. 10. A terminal basket closely encircles another chief cell. Gros method. × 650.



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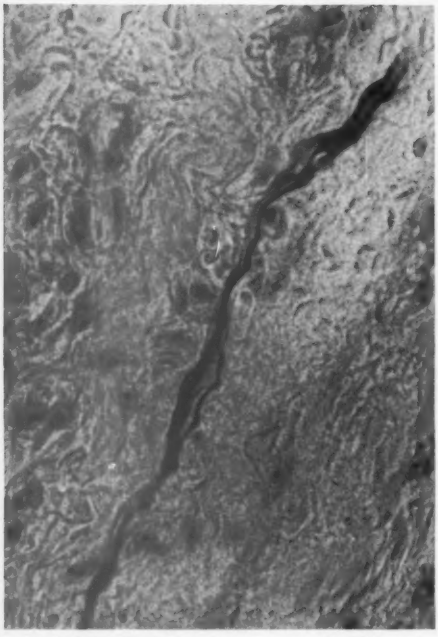

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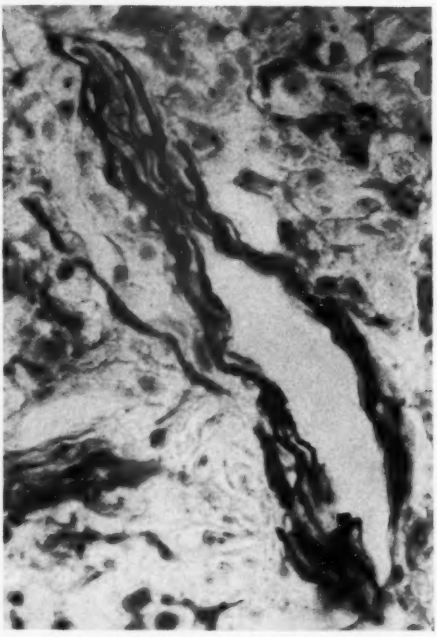

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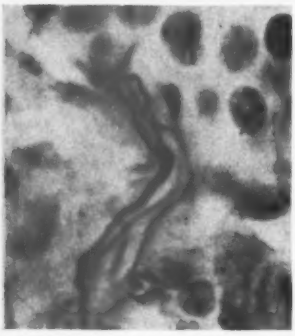

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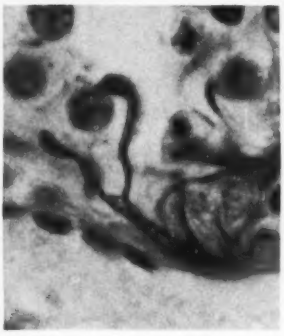

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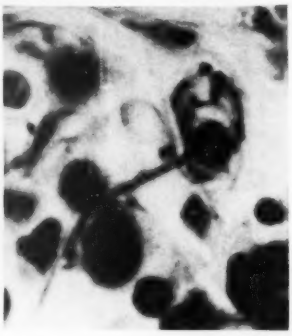

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PRIMARY POLYDIPSIA AND HYDRONEPHROSIS IN AN INBRED STRAIN OF MICE

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This paper describes a hereditary, apparently primary polydipsia and polyuria affecting more than 90 per cent of STR/N mice.[1] Individual animals have consumed up to 120 ml. of fluid, or 5 times their body weight, in 24 hours. In older males, the polyuria was complicated by massive hydronephrosis that developed in association with an obstructive plug in the lumen of the urethra. The STR/N mice were originally developed by Strong from the NHO strain by injection of 3-methylcholanthrene between the F_4 and F_{27} generations. They were transferred to the Inbred Colony of the National Institutes of Health in 1951 in the F_{28} generation for studies of gastric neoplasia. Although it will be shown that the syndrome probably existed in isolated individuals at that time, it has come to affect large numbers of animals only in recent years. The insatiable thirst of the mice does not correspond to other generally recognized disorders characterized by polydipsia. Two separate components of the syndrome have been investigated: (a) the nature of the polydipsia; and (b) the pathogenesis of the hydronephrosis.

MATERIAL AND METHODS

Animals

The initial observations were made on 255 STR/N males during other dietary studies[2] designed to carry the animals to 16 months of age. Simultaneous control observations were made on 419 mice of 3 other strains employed in these studies (DBA/2JN, 140; C57L/HeN, 175; and STR/1N, 104). Special studies were carried out, as listed in Tables I to VIII, on male and female STR/N mice at various ages and on several control strains: DBA/2JN, A/LN, A/HeN, BALB/cAnN, C57L/HeN, and STR/1N. The last mentioned strain, STR/1N, arose as a mutant of the STR/N and differs from it in coat color, in spontaneously developing obesity and in marked susceptibility to degenerative joint disease.

Housing and Diet

One to 5 mice were housed in plastic cages in a room maintained at 78° ± 2° F. The basic stock diet was Purina Laboratory Chow with water *ad libitum*. The special

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diets included: 10,046, containing 60 per cent vegetable fat; 10,046A, the same as the preceding but diluted with inert filler; 10,005A, Ingle's diet, that produced obesity by withholding drinking water except what was admixed with the food. The composition of these diets has been presented elsewhere.[8]

It is estimated that the amount of water each mouse ingested with the Ingle diet (10,005A) was 3.2 ml. per day. In effect, this regimen served as a restriction of water intake since the water consumed by the STR/N mice on an *ad libitum* intake at times reached 120 ml. A complete, purified diet (4,419A) with water *ad libitum*, also was fed to 30 STR/N mice; it did not affect the development of the syndrome, and therefore its results will not be described further.

Urinalysis

Methods for collecting urine directly from single mice and determining the specific gravity and osmolarity are described elsewhere.[8] Urine output was measured for periods up to 24 hours from 1 or more mice by means of glass metabolism cages which permitted the separate collection of urine and feces. Sodium concentration in the urine and plasma was determined by flame photometry.

Histologic Procedures

Serial histologic sections of the hypothalamic-pituitary gland region were made in 4 male STR/N and 2 control (A/LN and DBA/2JN) mice approximately 1 year old. In 1 polydipsic and 1 control mouse, the tissues were perfused via the right ventricle with Zenker's solution and sectioned in a horizontal plane parallel to the base of the brain. The blocks of tissue from these 2 animals were stained for neurosecretory substance by Gomori's chromalum-hematoxylin method.[4] In the other animals, the hypothalamic tissues were sectioned coronally and stained with aqueous toluidine blue or chromalum-hematoxylin. Sections of pituitary gland from 1 polydipsic STR/N and 1 control mouse were also stained by Glenner and Lillie's procedure.[5] Conventional hematoxylin and eosin-stained sections were made of adrenal, salivary, and thyroid glands, kidney, and pelvic tissues. Kidneys of several young STR/N mice prior to development of hydronephrosis were also examined for esterase activity of the vascular bundles by the naphthol AS-LC acetate method of Burstone.[9]

Experimental Manipulation

Details of the various procedures designed to elucidate the nature of the polydipsia (water deprivation, adrenalectomy, etc.) are presented in the tables and text. The hormonal preparations used were: pitressin tannate in oil (Parke, Davis & Company); DOCA (deoxycorticosterone acetate, Gotham Pharmaceutical Company), 5 mg. per ml.; ACTH (adrenocorticotropic hormone, National Drug Company) 20 units per ml.

Results

Description of the Syndrome

Occurrence and Mortality. Although polydipsia could be detected in 3-month-old STR/N mice fed a commercial stock diet by measuring the water intake (Table I), it became grossly obvious at 9 months of age. At that time 90 per cent of the animals were affected, as shown by the rapidity with which the water bottles emptied and the wood shavings became soaked with urine. The water consumed by unselected STR/N mice was approximately 6 times greater than the consumption by control animals (Table II). The mean urine output of the same animals was also much greater. Thus the mice with polydipsia imbibed, on the aver-

TABLE I

EFFECT OF AGE ON WATER INTAKE IN STR/N MICE

Age (mo.)	No. of cages measured *	Mean water intake †
3	12	9.4 ± 0.71 ‡
5-7	11	15.8 ± 2.0
8-9	7	32.4 ± 6.4
10	14	27.2 ± 2.2
11-16	13	30.6 ± 1.1

* One to 5 mice per cage, measured from 2 to 7 days; male.
† Ml. per mouse in 24 hours.
‡ Standard error of the mean.

TABLE II

WATER INTAKE AND URINE OUTPUT OF STR/N AND OTHER STRAINS OF MICE (MALES)

Strain	No. of groups *	Age (mo.)	Mean body wt. (gm.)	Mean water intake †	Mean urine output †
STR/N	4	11-14.5	31.4 ± 1.7 ‡	37.4 ± 6.8 ‡	14.1 ± 1.1 ‡
Control §	6	12-14	33.0 ± 4.1	5.9 ± 0.7	0.8 ± 0.3

* One to 4 mice per group.
† Ml. per mouse in 24 hours.
‡ Standard error of the mean.
§ STR/1N, BALB/cAnN, A/LN, DBA/2JN, C57/HeN.

age, more than their body weight in water each day, and in isolated instances the figure reached as much as 5 times this. Polydipsia, but not hydronephrosis, occurred to the same extent and severity in females and males (Table III). Of 83 male STR/N mice started on the commercial stock diet at weaning, only 17 (21 per cent) survived 16 months. In contrast, the survival rates in control strains were: C57L/HeN, 50 of

TABLE III

EFFECT OF WATER INTAKE AND URETHRAL PLUG ON HYDRONEPHROSIS AND MORTALITY IN MICE

Strain	Diet	Sex	Frequency of urethral plugs *	Water intake †	Hydro-nephrosis	Mortality
STR/N	Stock	M	14/14	28.2 ± 3.0 ‡	43/48 (89%)	66/83 (79%)
		F	0/11	25.2 ± 3.4	0/11 (0%)	0/11 (0%)
	10,005A	M	7/7	3.2 ± 0.2	0/25 (0%)	4/27 (15%)
Control §	Stock	M	26/28	5.3 ± 0.3	0/28 (0%)	Variable

* Frequency of urethral plugs based on animals whose water intake was measured (9 to 16 months old). Frequency of hydronephrosis based on those animals killed or not too autolyzed for examination when found dead. Mortality based on all animals 16 months of age.
† Ml. per mouse in 24 hours.
‡ Standard error of the mean.
§ BALB/cAnN, C57L/HeN, STR/1N, DBA/2JN, A/LN, C57BL/6JN.

53 (94 per cent); DBA/2JN, 25 of 33 (75 per cent); and STR/1N, 24 of 47 (51 per cent). The relatively low survival of the last strain resulted from a pelvic inflammatory disease (lower urinary tract) unrelated to the present syndrome.

The high mortality of the STR/N males was attributed to hydronephrosis since this was severe and was the only consistent gross alteration observed post mortem. Further evidence of this is provided by the absence of hydronephrosis in and by 100 per cent survival to 16 months by STR/N females with polydipsia (Table III).

Hydronephrosis. At necropsy, massive distention of the bladder, hydroureter and hydronephrosis were seen regularly in male STR/N mice dying spontaneously (Fig. 1). In advanced cases, the kidneys were converted into thin-walled sacs with little or no discernible parenchyma. As much as 22 ml. of urine (65 per cent of body weight) was recovered from the urinary tract in individual animals. In 9 males, the average volume of fluid in the urinary tract was 10.4 ± 1.5 (standard error) ml.; right kidney, 4 ± 1; left kidney, 1.8 ± 0.3; and bladder, 4.6 ± 0.4. Hydronephrosis was not present in 2 male STR/N mice dying at 4 and 7 months of age, but it was manifest in 33 of 34 males dying between 8 and 15 months, and in 10 of 14 males sacrificed at 16 months of age. In living animals, an enlarged bladder was palpable in 17 of 24 animals 10 months old and in each of 7 males surviving to 13 to 16 months. Enlarged kidneys were palpable in only 2 of the former and 5 of the latter age groups. Histologic sections of the distended urinary bladder and the hydronephrotic kidneys from several animals disclosed little or no inflammatory infiltration. In animals killed before the development of hydronephrosis, the kidneys were grossly and microscopically normal. Esterase activity of the type suggested to be associated with the medullary urinary concentrating apparatus in rats[7] was normal.

Urethral Plugs in Male Mice. The urethral lumen of older male mice of all strains contained a white, rubbery mass that conformed to the contours of the passage. The plugs occupied a variable proportion of the urethra. They usually extended proximally to the origin of the seminal ducts and sometimes through the bladder neck into the bladder (Fig. 2). The urethral plugs were not found in female mice. Plugs were not present prior to sexual maturation. Microscopically, the plugs were brightly eosinophilic and homogeneous, thus resembling seminal vesicle fluid.

The plugs in the STR/N mice were not larger than those of other strains (Table IV). Nevertheless, the absence of plugs in females and their failure to develop hydronephrosis in spite of the polydipsia indicates that the plugs acted as obturators in the STR/N males.

Hypothalamic, Endocrine, and Salivary Tissues. Abundant neuro-

secretory substance was present in the posterior pituitary lobe as well as the supra-optic nucleus and paraventricular fibers (Fig. 3). There was no distortion of the third ventricle. No areas of necrosis, loss of nuclei or gliosis of the hypothalamus were seen. The anterior pituitary lobes were normal histologically. The weights of the STR/N pituitary glands were similar to those of control strains of mice without polydipsia (Table IV). The adrenal glands of STR/N animals with polydipsia, not complicated by hydronephrosis, were comparable to those of con-

TABLE IV

WEIGHTS OF ADRENAL, PITUITARY AND URETHRAL PLUG IN STR/N AND OTHER STRAINS OF MICE *

Strain	No. of animals	Weight			
		Body (gm.)	Adrenal (mg.)†	Pituitary (mg.)	Urethral plug (mg.)
STR/N (no hydronephrosis)	6	31.9 ± 2.2 ‡	4.17 ± 0.2 ‡	1.49 ± 0.06 ‡	15.1 ± 0.5 ‡
STR/N (hydronephrosis)	2	40.0 ± 5.0	7.74 ± 1.34	1.50	10.9 ± 7.03
BALB/cAnN	4	30.5 ± 0.9	3.77 ± 0.09	1.52 ± 0.02	32.7 ± 0.9
C57L/HeN	3	25.0 ± 2.0	3.75 ± 0.62	1.52 ± 0.12	56.4 ± 8.6
STR/1N	1	45.5	5.12	1.70	3.02
DBA/2JN	6	29.4 ± 1.0	3.29 ± 0.12	1.58 ± 0.09	14.6 ± 5.1
A/LN	6	30.1 ± 0.3	3.60 ± 0.07	1.60 ± 0.07	18.3 ± 6.1
C57BL/6JN	6	28.9 ± 0.5	2.68 ± 0.09	1.80 ± 0.25	5.2 ± 2.6

* Nine months old, retired male breeders.
† Weight of both adrenal glands.
‡ Standard error of the mean.

trol mice (Table IV), but they became larger when hydronephrosis developed. Histologically, the adrenal glands were normal. The relative weights of the thyroid glands of the STR/N strain were high,[8] but other organ weights (lung, liver, spleen and nonhydronephrotic kidney) were not excessive. The salivary glands were normal histologically.

Urinalysis. The urine of STR/N mice was characteristically pale or colorless rather than amber-colored as in mice of strains without polydipsia. It was dilute and could be distinguished from urine of other strains at all ages (1 to 18 months) by its low specific gravity (Table V). The diluteness of the urine was also apparent from the low osmolarity, low sodium and total ionic concentration (Table VI). In severely affected animals, the specific gravity of the urine fell to values as low as 1.0035, compared to control values ranging from 1.031 to 1.130.[3] The mean osmolarity of the urine of STR/N mice was approximately ⅛ of the control values. The sodium and total ionic concentrations in the urine of the STR/N mice were much lower than in the controls (Table VI). No protein or reducing substance could be detected in the urine. Bacteria and unidentified mononuclear cells were present microscopically.

TABLE V

EFFECT OF 7-HOUR WATER DEPRIVATION ON URINE SPECIFIC GRAVITY AND BODY WEIGHT

Strain	Sex	No. of animals	Mean body wt. (gm.)	Loss of body wt. (%)	Mean urine specific gravity	
					Before water deprivation	After water deprivation
STR/N	F	6	31.1 ± 1.1 *	5.8 ± 1.2 *	1.010 ± 0.001 *	1.042 ± 0.003 *
	M	4	33.4 ± 1.3	4.5 ± 1.4	1.008 ± 0.003	1.023 ± 0.006
		3 †	45.6 ± 1.6	5.9 ± 2.5		
Control	F ‡	5	27.6 ± 1.2	4.8 ± 0.8	1.043 ± 0.007	> 1.080
	M §	4	33.3 ± 2.8	3.2 ± 0.8	1.051 ± 0.011	1.080

* Standard error of the mean.
† Three mice fed diet 10,046, high fat; all other mice fed stock diet.
‡ A/LN, BALB/cAnN.
§ A/LN, BALB/cAnN, STR/1N.

TABLE VI

PLASMA AND URINE OSMOLARITY, SODIUM CONCENTRATION AND IONIC CONCENTRATION IN STR/N AND CONTROL MICE *

Strain	Diet	Age	Osmolarity (mosm. per kg.)		Sodium (mEq/l.)		Ionic concentration (ppm.)	
			Plasma	Urine	Plasma	Urine	Plasma	Urine
Control †	Stock	6 mo.	327 ± 3 ‡ (6) §	1730 ± 95 ‡ (17)	146.97 ± 0.82 ‡ (6)	151.23 ± 26.44 ‡ (3)	7620 (2)	43,800 (1)
STR/N	Stock	6 mo.		684 ± 44 (6)				
		6 mo.	325 ± 10 (7)	298 ± 27 (24)	147.17 ± 1.07 (3)	15.45 ± 3.52 (8)	7960 (2)	3780 (2)
	4,419A			351 ± 28 (3)				
	10,046A			467 ± 44 (3)				
	10,005A			1906 ± 98 (4)				

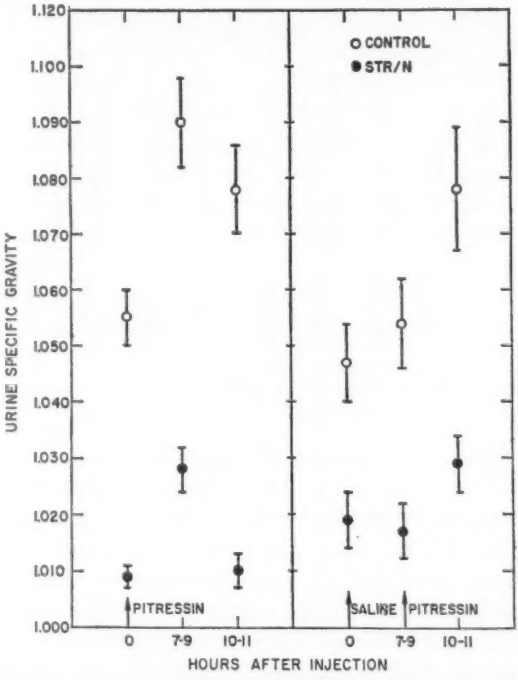
* Males and females.
† DBA/2JN, C57L/HeN, BALB/cAnN, (DBA/2JN x A/LN)F_1, (A/LN x DBA/2JN)F_1.
‡ Standard error of the mean.
§ Number of determinations; some of the plasmas are pooled from 2 mice.

Osmotic Concentration of Plasma. The plasma of STR/N mice with polydipsia was normal in osmolarity, sodium and total ionic concentrations (Table VI).

Experiments to Elucidate the Polydipsia

Effect of Pitressin. To determine whether lack of endogenous antidiuretic hormone or lack of renal response to it was responsible for the polyuria and polydipsia, a study was made of the effect of intramuscular

injection of pitressin on urine specific gravity and water intake of STR/N and control mice. The specific gravity of the urine of 6 STR/N mice, 7 to 9 hours following injection of the pitressin, increased from 1.009 to 1.024, while in the 6 control mice the corresponding values were 1.05 and 1.087 (Text-fig. 1). The specific gravity returned toward

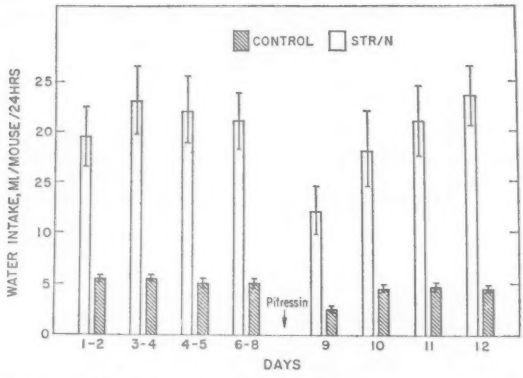


TEXT-FIGURE 1. Effect of injection of pitressin on the specific gravity of urine for 6 STR/N mice (both sexes) and controls, A/LN and DBA/2JN (both sexes). Each point represents the mean with the standard errors. The data on the left were obtained when the animals received water *ad libitum* while those on the right were obtained when another group of mice received injections of 0.05 ml. of saline at zero time; 0.25 pressor units of pitressin tannate in oil were injected intramuscularly.

the original figure 10 to 11 hours after the injection. When saline had been injected 7 hours previously, a similar increase in the specific gravity of urine from STR/N and control mice following administration of pitressin was seen (Text-fig. 1). Pitressin also reduced the water intake of the STR/N and the strains without polydipsia (Text-fig. 2) for a day or two. Nevertheless, in these experiments pitressin changed neither the water intake nor the urinary specific gravity in the STR/N mice to that of the strains without polydipsia.

Effect of Acute Deprivation of Water. Withholding drinking water

for 7 hours produced a sharp rise in urinary specific gravity in both STR/N and control mice (Table V). The degree of concentration achieved by STR/N mice under these conditions was not as great as that in the control animals, while the loss of body weight was as great or greater (Table V).



TEXT-FIGURE 2. Effect of injection of pitressin on consumption of water by STR/N and control mice (mean with standard errors).

Effect of Chronic Deprivation of Water. As already noted, the Ingle diet (10,005A) in effect restricted water intake to approximately 3.2 ml. a day. By this means hydronephrosis was completely prevented and the mortality reduced from 79 to 15 per cent at 16 months of age (Table III). The urine of the STR/N mice receiving diet 10,005A was much more concentrated (Table VI) than in the mice fed the commercial stock diet drinking water *ad libitum*. The osmolarity of the urine from STR/N mice receiving this limited quantity of water was comparable to that of control strains (Table VI) which drank similar amounts of water on an *ad libitum* basis (Table III). An even greater degree of urinary concentration was achieved by STR/N mice with more stringent restriction of water intake. Seventeen 2-month-old STR/N females were maintained for 68 hours on a diet of bread soaked in milk with no drinking water. At the end of that time the specific gravity of the urine exceeded 1.11 in 8 and averaged 1.094 in the other 9 mice. This corresponds to 3,700 and 3,160 mosm. per kg. of water.[3]

Thus the polydipsia was nonobligatory since the STR/N mice survived, conserved water and excreted a highly concentrated urine when water intake was restricted.

Effect of Adrenalectomy. The effect of bilateral adrenalectomy on fluid intake (water and 0.9 per cent saline) was determined in STR/N and control animals (Table VII). A daily intramuscular injection of

1 mg. of DOCA was given postoperatively to one group of adrenalectomized mice maintained on tap water. A second group received no DOCA but was given saline to drink for several days prior to and for 4 to 13 days following adrenalectomy. A third group of mice received water prior to, and saline following surgery. Sham operations were performed by comparable dorsolumbar body-wall incisions without removing the glands. Exploration for accessory adrenal tissue was not carried out, but ectopic glands were not apparent at the time of operation. The data are presented only for those mice surviving at least 2 days.

All STR/N mice showed an appreciable reduction in fluid intake following adrenalectomy while the control mice did not. Adrenalectomy, however, did not reduce the water intake to that of the controls. In a few STR/N mice, fluid intakes were comparable to those of STR/1N mice which became obese and had the highest water consumption of

TABLE VII

EFFECT OF ADRENALECTOMY ON FLUID INTAKE IN STR/N AND CONTROL MICE

Strain and no. of mice	Sex	Age (mo.)	Fluid consumption (ml.) Pre-operative Water	Pre-operative Saline	Postoperative Water	Postoperative Saline	DOCA	Days studied post-operatively
STR/N (3)	M	9–15	40.3 (28–54) *		19.0 (12–24)		+	5–18
STR/N (2) sham operated	M	9–10	26 (25–26)		34 (31–37)		+	6–8
subsequent adrenalectomy					17 (13–22)		+	10–11
STR/N (3)	M	9–11	20 (10–35)	27 (14–52)		22 (8–49)	—	4–13
STR/N (2) sham operated	M	10–11	20 (19–22)	21 (20–21)		16 (15–16)	+	7–8
STR/N (3)	F	12	29 (20–37)			17 (8–28)	—	2–9
STR/1N (4)	M	8–10	14 (11–17)		14 (11–17)		+	22–23
STR/1N (5)	M	8–12	14 (10–18)	23 (16–34)		21 (13–33)	—	12–17
C57L/HeN	M	7	8	12		12	—	8
BALB/cAnN (1)	M	14	8	6		8	—	10
BALB/cAnN (3)	F	12	9 (8–11)			11 (10–11)	—	7–8

* Range of values.

the control strains studied. The reduction in fluid intake was less marked when the animals had been primed with saline. Actually, the reduction in fluid intake of the latter mice (adrenalectomized) was no more than that observed in the sham-operated, saline-primed STR/N mice.

Effect of STR/N Plasma on Water Consumption in BALB/cAnN Mice. To see if a factor in the plasma of STR/N was responsible for the polydipsia, 1 ml. of fresh heparinized plasma from STR/N mice was injected intraperitoneally into each of 2 BALB/cAnN mice. Plasma from C57L/HeN mice without polydipsia was used as a control. The BALB/cAnN mice receiving injections of STR/N plasma showed no greater water consumption ($\Delta = 1.7$ ml. per mouse in 24 hours) than those receiving plasma from control (C57L/HeN) animals ($\Delta = 1.6$); the specific gravity of the urine 9 hours after injection of the plasma was reduced to the same extent in both groups ($\Delta = -0.006$ and -0.008 respectively).

Effect of Reserpine and Chlorpromazine. Since hypothalamic regions controlling water intake have been found in several species, the effect of reserpine and chlorpromazine on the polydipsia of STR/N mice was studied. These compounds block hypothalamic pathways,[9] and in rats, chlorpromazine depresses water intake.[10] Intramuscular injection of reserpine (0.3 mμg. per gm. of body weight) in 2 STR/N males resulted in a slight to moderate reduction of water intake, but failed to decrease it to nonpolydipsic levels. A similar result was obtained by the injection of chlorpromazine (1 μg. per gm. of body weight) in 1 STR/N and 1 control mouse. The possible nonspecific reduction of water intake resulting simply from needle puncture and endogenous release of pitressin was not controlled in this experiment; however, in another experiment, needle puncture with the injection of physiologic saline resulted in no increase of urinary specific gravity after 4 hours.

Effect of Dietary Fat. STR/N mice fed diets having a high content of vegetable fat, 10,046 (60 per cent) and 10,046A (37.4 per cent), showed a reduction of polydipsia, polyuria, the incidence of hydronephrosis and mortality (Table VIII). The osmolarity of the urine from STR/N mice receiving diets 10,046A with water *ad libitum* was somewhat greater than that of animals on the stock regimen (Table VI) but not as great as that in control strains without polydipsia.

Effect of Water Consumption ad Libitum After 15 Months of Restricted Intake. Diet 10,005A was fed to 8 STR/N mice from weaning to 16 months of age. These animals were restricted in water consumption since they received only the water mixed with the diet (about 3.2 ml. per mouse in 24 hours). At the end of the 16th month, water bottles were attached to the cages of these mice. Less than 30 minutes thereafter, the mice drank the water avidly and continuously for several minutes. Water intake rose from the estimated 3.2 ml. per day to 19 ml. by the third day, and to 30 ml. after 66 days. Concomitant with this, the specific gravity of the urine fell from 1.043 to 1.009 and 1.006 respec-

TABLE VIII

EFFECT OF DIETARY FAT ON WATER INTAKE AND URINE VOLUME IN STR/N MICE (MALES)

Diet		No.	Mean				
Type	Fat (%)	of mice	body wt. (gm.)	Mean water intake	Mean urine output *	Hydro-nephrosis †	Mortality †
Stock	6	11	34.4 ± 0.7 ‡	29.8 ± 3.6			
		6	28.5 ± 2.2	27.0 ± 4.0	14.3 ± 4.1	43/48 (89%)	66/83 (79%)
10,046	60	14	54.8 ± 1.1	12.6 ± 2.1			
		2	61.3 ± 14.1	23.0 ± 19.0	8.5 ± 1.5	10/32 (31%)	28/49 (57%)
10,046A	37.4	3	44.9 ± 3.8	11.6 ± 2.4			
		5	54.2 ± 3.7	54.2 ± 3.7	2.9 ± 0.5	4/23 (17%)	9/30 (30%)

* Ml. per animal in 24 hours.

† Mortality by 16 months of age based on all animals in the experiment; frequency of hydronephrosis based only on those animals killed or not too autolyzed when found dead.

‡ Standard error of the mean.

tively. However, the 8 STR/N mice thus allowed to imbibe water *ad libitum* had no hydronephrosis when killed 66 days later.

Nature of the Urethral Plugs. In an attempt to identify the plug with the seminal vesicle fluid, TRIS buffer extracts of this fluid and a urethral plug were made; chromatographic patterns of these extracts on a DEAF cellulose column at 4° C. showed that the proteins were not identical.[11] Two male STR/N mice in which orchiectomy was performed at 1 month of age had no urethral plug 4 months later, unlike all other males at a comparable age.

DISCUSSION

The STR/N mice have a genetic abnormality characterized by an extreme polydipsia and polyuria apparently serving no useful physiologic purpose. Actually, in male mice, the polyuria is responsible for a high mortality that can be overcome by restricting water intake. These animals have such a strong innate thirst that even though they have never been exposed to drinking water for periods as long as 15 months after weaning, they immediately and ravenously drink large volumes as soon as water becomes available.

There are 3 mechanisms that might explain polydipsia and polyuria: (a) excessive loss of water through the kidney; (b) disturbances in extra- and intracellular osmotic balance; and (c) excessive water intake resulting from a disturbed thirst regulation. The data from the present study suggest that the third possibility is the most likely one.

It is doubtful that polyuria was the primary pathologic process in this syndrome. This was indicated by the fact that when water consumption of the STR/N mice was restricted, they responded by excreting a concentrated urine and consequently did not die from dehydration. The

kidneys in these mice responded to pitressin as did those of control animals. There was no histologic evidence of intrinsic renal disease or of disturbance in the medullary urine-concentrating apparatus. The absence of reducing substances and the diluteness of the urine were evidence that a disturbance similar to diabetes mellitus was not involved.

The present data provide little direct evidence concerning a possible disturbance in extra- and intracellular osmotic balance. The plasma osmolarity of the STR/N mice receiving water *ad libitum* was the same as that in control mice. Unfortunately, we do not have comparable data for STR/N mice whose water intake was restricted. It is difficult to think that such an imbalance would permit normal growth and development over a period of 16 months or more.

Adrenal steroids are known to affect the rate and extent of water excretion[12] and also to alter the distribution of electrolytes in body tissue fluids. Adrenal cortical tumors produce a syndrome of polydipsia and polyuria in mice.[13] The size and structure of the adrenal glands were normal in STR/N mice with polydipsia uncomplicated by hydronephrosis. It may be assumed that the enlarged adrenal cortex associated with hydronephrosis was secondary to the obstructive uropathy. The diminution, albeit not abolition, of polydipsia in STR/N mice by adrenalectomy is difficult to evaluate. There was considerable variation in this response. When due allowance is made for the general debility of these animals following adrenalectomy and for the possibility that ectopic cortical tissue may have been overlooked, the fact that there was no uniform suppression of polydipsia is reasonable evidence that the polydipsia was not the result of primary hyperadrenocorticism.

For the above reasons and because of the apparent thirst of the water-restricted STR/N mice (fed ration 10,005A), it would appear that the polydipsia is the primary factor in the present syndrome. The mechanisms for regulating thirst include central (hypothalamic) and peripheral (oral and gastric) osmoreceptors.[9] In the STR/N mice there was no histologic evidence of hypothalamic or posterior pituitary abnormalities; they both contained abundant neurosecretory substances. The normal serum osmolarity of the STR/N mice is in keeping with the histologic evidence of abundant neurosecretory material within the neurohypophysis. The action of pitressin in reducing water consumption and producing a more concentrated urine cannot be interpreted as evidence of insufficiency of endogenous antidiuretic hormone production. The dose of pitressin used was enormous—about 3,000 to 5,000 times the minimum effective intravenous dose (0.05 m.u. per 20 gm. of mouse[14]), albeit administered intramuscularly in an oily menstruum. It caused a similar response in strains without polydipsia. Although pitressin is be-

lieved to influence urine volume through its action on the distal tubule,[15] there is some evidence that it also affects osmoreceptors.[16]

Some of the hypothalamic osmoreceptors appear to be related, spatially at least, to the appetite centers.[17–19] It is therefore of interest that the STR/N mice are closely related genetically to STR/1N mice that become obese spontaneously and have the highest water consumption of the strains with polydipsia studied. Chlorpromazine, which depresses food and water consumption in rats,[10] presumably by acting on hypothalamic centers, reduced but did not abolish the polydipsia in STR/N mice; reserpine did likewise. In the STR/N mice, the salivary glands, which have at times been implicated in the regulation of water intake,[16] were normal histologically.

Special studies of the stomachs of these mice were not made, but it is noted that the STR/N mice were originally developed for investigations of gastric neoplasm. The necropsy records shortly after the strain was received at the National Institutes of Health in 1951–1952 noted massive hydronephrosis in 2 of 52 STR/N males dying after 1 year of age. Thus, the trait was present in this stock years ago and apparently was inbred fortuitously into successive generations until it came to affect more than 90 per cent of the animals. Since 3-methylcholanthrene was used in developing the STR/N strain, one might speculate about the role of the carcinogenic agent in inducing the anomalous pattern of water consumption.

The severity of the polydipsia was influenced to a certain extent by the type of diet fed the animals. The greatest water consumption by STR/N mice occurred when they were fed the stock diet. The large water intake was not a unique result of this diet since a comparable polydipsia occurred in mice fed a purified diet of low caloric density (4,419A). The water consumed by the STR/N mice fed the high fat diet (10,046 and 10,046A) was considerably less than that of the animals fed the stock diet. When high fat diets are consumed, the oxidation of the foodstuffs produces a larger amount of water than when low fat diets are used. On this basis it can be argued that the mice fed the high fat diets secured more metabolic water than those fed the low fat diets and this, in turn, should reduce their water intake. This is, however, not the sole explanation for the action of the high fat diet in reducing the polydipsia since there was a concomitant reduction in urine volume. Actually, the reduction in urine volume was great enough to produce a reduction both in the occurrence of hydronephrosis and in mortality.

The hydronephrosis in male STR/N mice appeared adequately accounted for by the polydipsia which produced large urine volumes. The plugs in the urethral passage impeded the movement of the large vol-

umes of urine in the mice with polydipsia. Comparable plugs occur in males of other strains, but they presumably do not lead to kidney abnormalities since mice of other strains have small urine volumes. The plug appeared essential for the development of hydronephrosis, since in females, where the plugs were not present, the polydipsia did not lead to the kidney disturbance. The plugs presumably were of seminal origin because they did not appear in mice which had had orchiectomy. The proteins in the plug may have differed from those in the seminal fluid as a result of (a) accretion of other substances such as spermatozoa and secretions of the coagulating and other glands; (b) modification of the plug proteins by the preceding; and (c) leaching out of certain plug proteins by urine.

The various experimental manipulations performed to elucidate the genesis of the polydipsia and polyuria disclosed no appreciable difference in the response of the STR/N mice from those of the controls with the possible exception of adrenalectomy. The condition manifests itself in the STR/N mice by an apparently overwhelming desire to drink. In view of these observations, we can only speculate as to the ultimate cause of the abnormality.

Summary

A genetic abnormality manifested by an extreme polydipsia and polyuria that seemed to serve no useful physiologic purpose has appeared in STR/N mice. In males, the polyuria led to hydronephrosis apparently because a plug in the urethra impeded the passage of the large volumes of urine. These plugs were present also in males of other strains where they produced no disturbance since urine volumes were normal. STR/N females had polyuria but, in the absence of the plugs, did not develop hydronephrosis.

The urine excreted by mice with polydipsia had a very low specific gravity. Although the osmolarity, conductivity, and sodium concentration of the urine was much lower than that of control mice, there were no differences in the plasmas. The kidneys responded to pitressin with a reduction in urine volume and an increase in its concentration; a similar response was obtained by water deprivation. There were no histologically demonstrable abnormalities in the hypothalamus or pituitary glands. Adrenalectomy, like the injection of reserpine or chlorpromazine, reduced somewhat but did not abolish the polydipsia.

The consumption of water *ad libitum* was decreased when the STR/N male mice were fed a diet high in fat, and with this there was a reduction in mortality. Water restriction for periods as long as 15 months eliminated the polyuria and the hydronephrosis, but had no effect on thirst,

as evidenced by the compulsive drinking when these mice were given water *ad libitum*.

References

1. Carter, T. C.; Dunn, L. C.; Falconer, D. S.; Grüneberg, H.; Heston, W. E., and Snell, C. D. Standardized nomenclature for inbred strains of mice. *Cancer Res.*, 1952, **12**, 602–613.
2. Sokoloff, L.; Mickelsen, O.; Silverstein, E.; Jay, G. E., Jr., and Yamamoto, R. S. Experimental obesity and osteoarthritis. *Am. J. Physiol.*, 1960, **198**, 765–770.
3. Silverstein, E. Urine specific gravity and osmolality in inbred strains of mice. *J. Appl. Physiol.*, 1961, **16**. (In press.)
4. Lillie, R. D. Histopathologic Technic and Practical Histochemistry. The Blakiston Co., New York, 1954, p. 188.
5. Glenner, G. G., and Lillie, R. D. A rhodocyan technic for staining the anterior pituitary. *Stain Technol.*, 1957, **32**, 187–190.
6. Burstone, M. S. The cytochemical localization of esterase. *J. Nat. Cancer Inst.*, 1957, **18**, 167–172.
7. Longley, J. B.; Banfield, W. G., and Brindley, D. C. Structure of the rete mirabile in the kidney of the rat as seen with the electron microscope. *J. Biophys. & Biochem. Cytol.*, 1960, **7**, 103–106.
8. Silverstein, E.; Sokoloff, L.; Mickelsen, O., and Jay, G. E., Jr. Thyroid function in various strains of mice as measured by T/S ratio, plasma protein-bound iodine and thyroid weight. *Am. J. Physiol.*, 1960, **199**, 203–208.
9. Unna, K. R. A review of the neurophysiological effects of psychotherapeutic drugs. *Ann. New York Acad. Sc.*, 1957, **66**, 777–783.
10. Schmidt, H., Jr. Pentobarbital facilitation of water ingestion in the albino rat. *J. Comp. & Physiol. Psychol.*, 1958, **51**, 26–28.
11. Sober, H. A., and Peterson, E. A. Chromatography of proteins on cellulose ion-exchangers. (Letter to the editor.) *J. Am. Chem. Soc.*, 1954, **76**, 1711–1712.
12. Gaunt, R.; Birnie, J. H., and Eversole, W. J. Adrenal cortex and water metabolism. *Physiol. Rev.*, 1949, **29**, 281–310.
13. Bahn, R.; Wilson, H.; Kedda, L.; Furth, J., and Anderson, E. Metabolic changes associated with transplantable ACTH-producing pituitary tumors of the mouse. *Canad. J. Biochem. & Physiol.*, 1957, **35**, 1057–1065.
14. Heller, H., and Blackmore, K. E. The assay of small amounts of antidiuretic activity by intravenous injections into mice. *J. Endocrinol.*, 1952, **8**, 224–228.
15. De Wardener, H. E. Polyuria. *J. Chronic Dis.*, 1960, **11**, 199–212.
16. Wolf, A. V. Thirst. Physiology of the Urge to Drink and Problems of Water Lack. Charles C Thomas, Springfield, Ill., 1958, 546 pp.
17. Morrison, S. D., and Mayer, J. Adipsia and aphagia in rats after lateral subthalamic lesions. *Am. J. Physiol.*, 1957, **191**, 248–254.
18. Montemurro, D. G., and Stevenson, J. A. F. The localization of hypothalamic structures in the rat influencing water consumption. *Yale J. Biol. & Med.*, 1955–1956, **28**, 396–403.

19. ANAND, B. K., and DUA, S. Hypothalamic control over water consumption in the rat. *Indian J. M. Res.*, 1958, **46**, 426–430.

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LEGENDS FOR FIGURES

FIG. 1. Male STR/N mouse, showing bladder (reflected downward), ureters and kidneys greatly distended with urine. As is invariably the case, the right kidney is more enlarged than the left. The adrenal glands are also enlarged.

FIG. 2. White plug lying in the urethral lumen and extending proximally into the bladder.

FIG. 3. Horizontal section of the supraoptic nucleus in a male STR/N mouse. Dark-staining neurosecretory substance is seen in axons and cell bodies. Chromalum-hematoxylin stain. Approximately × 500.

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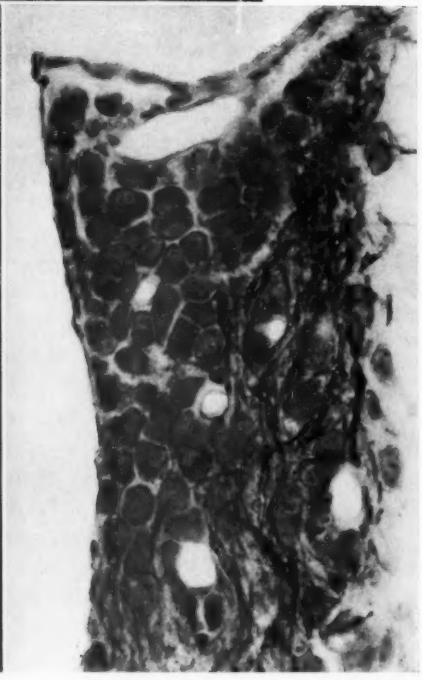

3

THE CYTOCHEMISTRY OF ANOXIC AND ANOXIC-ISCHEMIC ENCEPHALOPATHY IN RATS

I. Alterations in Neuronal Lysosomes Identified by Acid Phosphatase Activity

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There has been renewed interest in the acid hydrolases in view of the lysosome concept formulated in 1955 by de Duve.[1,2] This concept suggests that many hydrolytic enzymes, all with pH optimum in the acid range, are enclosed within a cytoplasmic organelle—the lysosome—delimited by a single lipoprotein membrane. This membrane restricts the freedom and substrate accessibility of its enclosed enzymes. *In vivo* changes in relation to enzyme and substrate have been suggested for phagocytosis, pinocytosis and various lytic processes.

Originally defined in biochemical terms, the role of lysosomes in necrobiosis has been described in morphologic terms by Novikoff.[3-5] Lysosomes were identified in tissue sections by virtue of their acid phosphatase activity. Abnormal variations in both the number and size of the lysosomes were noted in renal infarcts, hydronephrosis, and in liver, following common bile duct ligation. The term "cytolysome" was coined to describe abnormally large lysosomes found in the necrobiotic cells.

While the applications of the lysosome concept to neurobiology are relatively unexplored, the enzymatic activity used in their identification (acid phosphatase) has been the subject of numerous earlier reports.[6-11] In these studies, the Gomori lead procedure for acid phosphatase[12] was applied to acetone- or formalin-fixed, or frozen-dried tissue embedded in paraffin. In general, neuronal activity was characterized by nuclear or nucleolar staining associated with variable and usually diffuse staining of the cytoplasm. In most instances, the staining data provided by these earlier methods of fixation and embedding have been difficult to translate into enzyme localization due to the prevalence of adsorption artifacts.[13] In addition, the morphologic preservation of many intracellular organelles (e.g., lysosomes) was inadequate.

Recently, one of us[14] has described cytologic and histologic localization of several enzyme activities that survive cold formol-calcium fixa-

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tion in rat brain. The advantage of this fixative is that it preserves morphologic detail while permitting visualization of such enzyme activities as DPNH- and TPNH-tetrazolium reductases in mitochondria and basophilic substance, adenosine triphosphatase in cell membranes and acid phosphatase in lysosomes.

The purpose of this investigation is to describe alterations in neuronal lysosomes of the rat brain during postmortem autolysis and in anoxic and anoxic-ischemic encephalopathy. The classic neurohistologic techniques have been of limited value in detecting the early phases of neuronal degeneration. Any pathologic alteration in lysosomes might be a useful index of early neuronal necrobiosis.

Most anoxic lesions in rats [15–17] have been difficult to produce, are small in size, and therefore not well suited for exploratory cytochemical studies. Recently, Levine [18] has described a method capable of producing anoxic lesions in the rat brain by combining anoxic anoxia (a single exposure to nitrous oxide or nitrogen) and ischemia (unilateral common carotid artery ligation). This method results in extensive lesions of both the white and gray matter of forebrain structures in over 80 per cent of the animals. Thus, an experimental cerebral lesion suited for cytochemical investigation is now available.

Methods

Both male and female Sprague-Dawley rats, weighing 150 to 250 gm., were used in all the experiments. They were killed by crushing the cervico-thoracic spinal cord; the cranial vault was rapidly reflected and the intact brain removed. The brain was then divided into 2 or 3 mm. thick coronal sections and placed in ice cold formol-calcium.[19]

Postmortem Autolysis

Brains were first sectioned in half longitudinally. One half, cut into coronal sections, was placed directly in the fixative. Before fixation the remaining half was placed in a sterile Petri dish and incubated at 37° C. for periods ranging from 5 minutes to 24 hours.

Anoxic-Ischemic Encephalopathy

Animals were subjected to the procedure of Levine [18] by means of nitrogen as the anoxic agent. Anoxic exposure was initiated 40 to 60 minutes after ligation of the left common carotid artery under ether anesthesia. The animals were maintained in a relatively stable unconscious state for periods of 30 to 60 minutes. During this period depressed respiratory rates of 25 to 50 per minute were observed. Following this anoxic episode, animals were allowed to survive for periods ranging from immediate sacrifice to 8 days.

Anoxic Encephalopathy

Three different schedules of anoxic exposure were used.

One group was exposed to a continuously flowing atmosphere of 100 per cent nitrogen for 60 to 90 seconds. Exposure ended when apnea supervened. These animals were killed at 30 minutes, 6 and 24 hours after exposure.

In the second group 3 such acute exposures were given 1 hour apart. The animals were sacrificed 4, 6 and 24 hours after the last exposure.

The third group was placed in a continuously flowing atmosphere consisting of 8 per cent oxygen and 92 per cent nitrogen containing traces of carbon dioxide. Increased bursts of nitrogen were given at hourly intervals so that oxygen levels were lowered sufficiently to produce severe agitation and respiratory distress. The rats were exposed to this environment in 4 hour daily periods and sacrificed after the second, third and fourth exposure days.

Control Groups

Normal rat brains were processed during these experiments as controls. In another control group, rats given 15 minutes of deep ether anesthesia or whose common carotid artery was ligated without further anoxia were killed 4 to 6 hours after the respective procedures.

Histologic Technique

The blocks were allowed to fix for 18 to 20 hours in cold formol-calcium (2 to 4° C.). Frozen sections, 10 to 20 μ thick, were prepared on a Spencer sliding freezing microtome.

The sections were rinsed in distilled water and stained for acid phosphatase activity by the method of Gomori.[13] Sections were incubated for 30 minutes at 37° C. in an appropriate volume of the following mixture: 500 ml. of 0.05 M acetate buffer, pH 5; 600 mg. of lead nitrate; and 50 ml. of 3 per cent sodium glycerophosphate (25 per cent alpha, 75 per cent beta). They were then rinsed in distilled water, placed in dilute ammonium sulfide for 1 to 2 minutes, washed well in distilled water and mounted in glycerogel. In all cases substrate control sections were incubated in the absence of substrate (glycerophosphate).

Unfrozen blocks of tissue, adjacent to those taken for frozen sections, were embedded in paraffin. Sections 6 to 8 μ thick were stained with hematoxylin and eosin or thionine ("paraffin Nissl").

Results

Normal and Control Animals

In addition to 25 normal brain halves derived from the autolysis experiments, the brains of 15 normal animals also were available for examination.

As previously noted,[14] neuronal acid phosphatase activity was localized in the lysosomes. These discrete organelles, 0.3 to 0.7 μ in diameter, were blackened by accumulated lead sulfide and stood out prominently against an unstained cytoplasmic background (Figs. 1 to 3). With some experience, a normal base line was established, against which pathologic alterations in the lysosomes were compared. Thus, in normal rats, the Purkinje cells (Fig. 1) and the larger cerebral cortical neurons (Fig. 2) contained larger lysosomes, with greater intracellular variation in their size and number, than the smaller neurons of the cerebral cortex (Fig. 3) or the pyramidal band of the hippocampus. The staining was reproducible and distinct. Nuclear and nucleolar staining was usually minimal and inconstant. All substrate control sections were negative.

No histochemical or histologic abnormalities were present in the brains of 3 animals given ether anesthesia only, or in 3 brains obtained after

unilateral common carotid artery ligation without further anoxic treatment.

Autolysis

In sections stained with the Nissl method and hematoxylin and eosin, no abnormalities were noted earlier than 40 minutes (8 animals). From 40 minutes to 12 hours (16 animals) autolysis became increasingly severe. These changes were particularly evident in the cerebellum, cerebral cortex and corpus striatum. At 24 hours (1 animal), almost complete homogeneation of all cell structures was seen.

In sections stained for acid phosphatase activity, no abnormalities were noted earlier than 10 minutes. From 10 minutes to 1 hour, progressive swelling of the lysosomes ("cytolysome" formation) was noted. This was associated with frequent clumping and gradual reduction in the total number of lysosomes (Fig. 4). The cytolysomes appeared earliest in the Purkinje cells and layers 5 and 6 of the neocortex. After 1 hour, progressive loss of both lysosomes and cytolysomes occurred so that at 8 hours (Fig. 5) few cytoplasmic sites of activity remained. Nuclear staining became prominent. The large neurons of the corpus striatum and layers 5 and 6 of the neocortex were relatively resistant to the loss of enzyme activity when compared with the Purkinje cells and the neurons of the hippocampus, thalamus and layers 1 to 3 of the neocortex. From 8 hours onward, considerable deposition of nonspecific lead sulfide appeared in the substrate control sections.

Anoxic-Ischemic Encephalopathy

Of the 53 rats subjected to the anoxic-ischemic procedure, 17 died during or after the period of anoxia. A clinically evident hemiparesis was present in 20 of the surviving 36 animals examined.

The neuronal lesions were similar in extent and distribution to those described by Levine.[18] Lesions were noted in 9 of 12 animals allowed to survive 24 hours and in 6 of 18 animals in the 1 to 12 hour survival group. No histologic lesions were present in the 6 animals killed immediately after the anoxic period. Of interest was the observation of focal neuronal damage in the caudate nucleus of an animal surviving 3 hours and in the hippocampus and neocortex of 2 animals surviving 4 to 6 hours.

Cytochemical studies revealed striking alterations in lysosomes. In many cases the histologic and cytochemically treated sections were in such proximity that tissue from the same injured zone could be compared (Figs. 10 and 11). Particularly impressive was the presence of neuronal cytolysomes in 2 of the 6 rats killed immediately after the anoxic period.

In 1 of these rats, clusters of neurons with prominent cytolysomes were present in layers 4 and 5 of the neocortex (Fig. 6), the pyriform cortex (Fig. 7), and the pyramidal band of neurons in the hippocampus. Occasional cytolysomes were present in the Purkinje cells. In the second animal, zones with less extensive alteration of neurons were observed in the pyriform cortex and hippocampus. Occurring with greatest frequency on the side of the carotid ligation, occasional foci of similarly altered neurons were present in the contralateral pyriform cortex and hippocampus. Similar alterations in lysosomes were observed in 15 of the 18 animals surviving 1 to 12 hours. Again, clusters of neurons in the pyriform cortex, hippocampus (Figs. 8 and 9) and neocortex were most frequently affected. A few cytolysomes were present in the Purkinje cells and neurons of the corpus striatum and thalamus. Bilateral lesions were present in 5 animals.

Where cytolysome formation was striking, the overall number of lysosomes in that cell appeared reduced. Many of the affected neurons appeared swollen. The largest cytolysomes were seen in the pyriform cortex and hippocampus where they were frequently as large as 3 to 4 μ in diameter. Present in all portions of the neuron, the cytolysomes appeared with greatest frequency at the base of the apical processes.

Histochemical changes were present in 9 of the 12 rats allowed to survive over 24 hours. These lesions were localized to zones of advanced degeneration and neuronal loss (Fig. 10). In 5 rats the lesions were bilateral.

In contrast to the early lesions which displayed prominent cytolysome formation with minimal loss of remaining lysosomes, the neurons in the older infarcts revealed a more pleomorphic cytochemical pattern (Fig. 11). In many neurons, large cytolysomes could still be seen. However, a large proportion of the neurons were now devoid of both cytolysomes and lysosomes. In many of the shrunken neurons, the nuclei and nucleoli were deeply stained. In some infarcts only a few cytolysomes remained; the predominant picture was that of loss of acid phosphatase activity. Purkinje cell cytolysomes were present in only 2 animals in this late survival group.

In almost half of the advanced infarcts, control sections incubated in the absence of substrate possessed fine granules of lead sulfide. These positive substrate controls were found only in the group surviving 24 hours or longer.

Anoxic Encephalopathy

In the first group, 13 of 16 animals survived the acute exposure to 100 per cent nitrogen. In only one rat of 4 surviving 30 minutes were

focal collections of neurons with cytolysomes seen. These were found in layers 3 and 4 of the neocortex and in the Purkinje layer. Of the 6 animals surviving 6 hours, cytolysomes were present in Purkinje cells of 2 rats and in the Purkinje cells and neocortical neurons of a third. Prominent cytolysomes were present in the Purkinje cells, hippocampus and neocortex of all 3 rats surviving 24 hours. This was associated with early acute cell changes in Purkinje cells (Nissl stains) of 2 of these animals.

In the second group, 7 of 13 animals survived the repeated exposures to nitrogen. In 2 of 4 rats sacrificed between 4 and 6 hours, cytolysomes were identified in both the pyriform and neocortex (Fig. 12) and in the Purkinje cells. Cytolysomes were present in the neocortex and Purkinje cells of all 3 animals surviving 24 hours. Paraffin sections of this tissue were normal.

In the third group, 3 of 5 animals survived the schedule of prolonged critical hypoxia. In all 3 survivors prominent cytolysomes were present in the neurons of the pyriform cortex, neocortex and hippocampus. In 2 rats, cytolysomes were present in Purkinje cells (Fig. 13). Again, paraffin sections appeared normal.

Discussion

No attempt has been made to describe in detail the anatomic localizations of the various lesions. Rather, it has been our purpose to correlate the natural history of the lysosome with the known processes of neuronal autolysis and necrobiosis. Significant and easily detectable morphologic alterations in neuronal lysosomes were seen in both autolysis and necrobiosis. In both events, the changes were similar and consisted of early swelling of the lysosomes ("cytolysome" formation) followed by gradual loss of remaining lysosomes and, ultimately, by loss of all sites of acid phosphatase activity.

It was difficult to determine whether cytolysome formation was a potentially reversible lesion. The association of numerous cytolysomes in early lesions with less impressive late neuronal loss (Nissl stains) would support such a view. Nevertheless, many cytolysomes could still be seen in zones of obviously irreversible damage. Considerable loss of enzyme activity was seen only in the late phases of neuronal degeneration.

The similarity of the anoxic and autolytic cytochemical patterns necessitated the use of fresh tissue if early necrobiotic changes were to be evaluated. Nevertheless, the lysosome remained a most sensitive index of neuronal damage. In addition, there was no correlation between the cytochemical alterations and the frequent occurrence of "dark cell" neurons in the Nissl stains which represented, for the most part, fixation

artifacts. The cytochemical changes occurred earlier and with greater frequency than any lesions encountered in conventional stains.

It is reasonable to assume that the cytochemically visualized sites of acid phosphatase activity probably correspond to the particulate, bound (latent) cell fractions of the biochemist. The free (solubilized, unsedimentable) fraction of enzyme would not be detected in the tissue sections because of diffusion from the cells. The overall pathologic fate of the neuronal lysosome correlates well with biochemical studies of ischemic necrosis and autolysis in liver. Marked increases in free acid phosphatase activity (with corresponding reduction in bound activity) occurs within 60 minutes of autolysis [20] and within 6 hours after the onset of ischemic necrosis.[2]

The function of the lysosome in the metabolic economy of the cell remains speculative. The morphologic observations of Novikoff [4,5] suggest a close relationship of the lysosome to, and perhaps an identity with, pinocytosis vacuoles. Biochemical studies of lysosome fractions have revealed, in addition to acid phosphatase, acid ribonuclease, acid deoxyribonuclease (DNase II), phosphoprotein phosphatase, cathepsin, beta glucuronidase, arylsulfatases A and B and beta-N-acetyl-glucosamidase.[21] Lysosomal enzymes are capable of destroying most of the important cell constituents. Indeed, the ribonuclease activity in these organelles suggests a causal relationship between the lysis of neuronal lysosomes and subsequent chromatolysis. It is likely that all necrobiotic phenomena are ultimately mediated through these organelles. The conditions under which the integrity of the lysosomal membrane is disrupted *in vivo* and its lytic enzymes activated are not entirely clear. Therefore, morphologic and biochemical changes in lysosomes may prove useful in detecting the early effects of toxic, chemotherapeutic and radiobiologic agents. It is evident that the lysosome now looms as an organelle of unquestioned physiologic significance, whose application to pathology deserves further exploration.

Summary

Alterations in neuronal lysosomes during postmortem autolysis and anoxic and anoxic-ischemic encephalopathy have been described. The lysosomes were identified in formol-calcium fixed tissue by virtue of their acid phosphatase activity.

Enlargement and clumping of the lysosomes ("cytolysome" formation) were noted during the early phases of both autolysis and necrobiosis. Loss of lysosomes became prominent during the later phases.

In all cases the earliest cytochemical changes preceded those which could be detected by Nissl and hematoxylin and eosin stains.

References

1. De Duve, C.; Pressman, B. C.; Gianetto, R.; Wattiaux, R., and Appelmans, F. Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat liver tissue. *Biochem. J.*, 1955, **60**, 604–617.
2. De Duve, C. Lysosomes, a New Group of Cytoplasmic Particles. In: Subcellular Particles. A Symposium Held During the Meeting of the Society of General Physiologists at the Maine Biological Laboratory, Woods Hole, Mass., June 9–11, 1958. Hayashi, T. (ed.) Ronald Press, New York, 1959, pp. 128–159.
3. Novikoff, A. B. The proximal tubule cell in experimental hydronephrosis. (Brief notes) *J. Biophys. & Biochem. Cytol.*, 1959, **6**, 136–138.
4. Novikoff, A. B. Lysosomes and the physiology and pathology of cells. *Biol. Bull.*, 1959, **117**, 385.
5. Novikoff, A. B. Biochemical and Staining Reactions of Cytoplasmic Constituents. In: Developing Cell Systems and Their Control. Rudnick, D. (ed.) Ronald Press, New York. (In press.)
6. Wolf, A.; Kabat, E. A., and Newman, W. Histochemical studies on tissue enzymes. III. A study of the distribution of acid phosphatases with special reference to the nervous system. *Am. J. Path.*, 1943, **19**, 423–439.
7. Smith, W. K. The results of application of the "acid phosphatase" method to nervous tissue after formalin fixation. *Anat. Rec.*, 1948, **102**, 523–532.
8. Shimizu, N. Histochemical studies on the phosphatase of the nervous system. *J. Comp. Neurol.*, 1950, **93**, 201–217.
9. Naidoo, D., and Pratt, O. E. The localization of some acid phosphatases in brain tissue. *J. Neurol. Neurosurg. & Psychiat.*, 1951, **14**, 287–294.
10. LaVelle, A.; Liu, C. N., and LaVelle, F. W. Acid phosphatase activity as related to nucleic acid sites in the nerve cell. *Anat. Rec.*, 1954, **119**, 305–323.
11. Bodian, D., and Mellors, R. C. The regenerative cycle of moto-neurons, with special reference to phosphatase activity. *J. Exper. Med.*, 1945, **81**, 469–488.
12. Gomori, G. Microscopic Histochemistry; Principles and Practice. University of Chicago Press, Chicago, 1952, 273 pp.
13. Novikoff, A. B. Histochemical and Cytochemical Staining Methods. In: Analytical Cytology. Methods for Studying Cellular Form and Function. Mellors, R. C. (ed.) The Blakiston Div., McGraw-Hill Book Co., Inc., New York, 1955, ed. 1, pp. 2/1–2/63.
14. Becker, N.; Goldfischer, S.; Shin, W., and Novikoff, A. The localization of enzyme activities in rat brain. *J. Biophys. & Biochem. Cytol.* (In press.).
15. Yant, W. P.; Chornyak, J.; Schrenk, H. H.; Patty, F. A., and Sayers, R. R. Studies in asphyxia. IV. Neuropathology resulting from comparatively rapid asphyxia by atmospheres deficient in oxygen. *Pub. Health Bull.*, 1934, **211**, 29–40.
16. Lucas, B. G. B. Anoxia and the central nervous system: an experimental and clinical study. *Thorax*, 1946, **1**, 128–142.
17. Hicks, S. P. Brain metabolism *in vivo*. 1. The distribution of lesions caused by cyanide poisoning, insulin hypoglycemia, asphyxia in nitrogen and fluoroacetate poisoning in rats. *Arch. Path.*, 1950, **49**, 111–137.
18. Levine, S. Anoxic-ischemic encephalopathy in rats. *Am. J. Path.*, 1960, **36**, 1–17.

19. BAKER, J. R. The histochemical recognition of lipine. *Quart. J. Micr. Sc.*, 1946, 87, 441–470.

20. VAN LANCKER, J. L., and HOLTZER, R. L. The release of acid phosphatase and beta-glucuronidase from cytoplasmic granules in the early course of autolysis. *Am. J. Path.*, 1959, **35**, 563–573.

21. DE DUVE, C. The function of intracellular hydrolases. *Exper. Cell Res.*, 1959, Supp. 7, 169–182.

The authors wish to acknowledge the helpful criticisms of Drs. Harry M. Zimmerman and Alex B. Novikoff. The photomicrographs were prepared by Messrs. J. Walker and A. Hershkowitz.

[*Illustrations follow*]

Legends for Figures

Except where indicated, all photographs were made from sections prepared to demonstrate acid phosphatase activity.

Figures 1 to 3 show the distribution of lysosomes in the normal rat brain. The lysosomes are stained black because of the deposition of lead sulfide. Note the greater variations in the size and number of neuronal lysosomes in Figures 1 and 2 when compared with those in Figure 3.

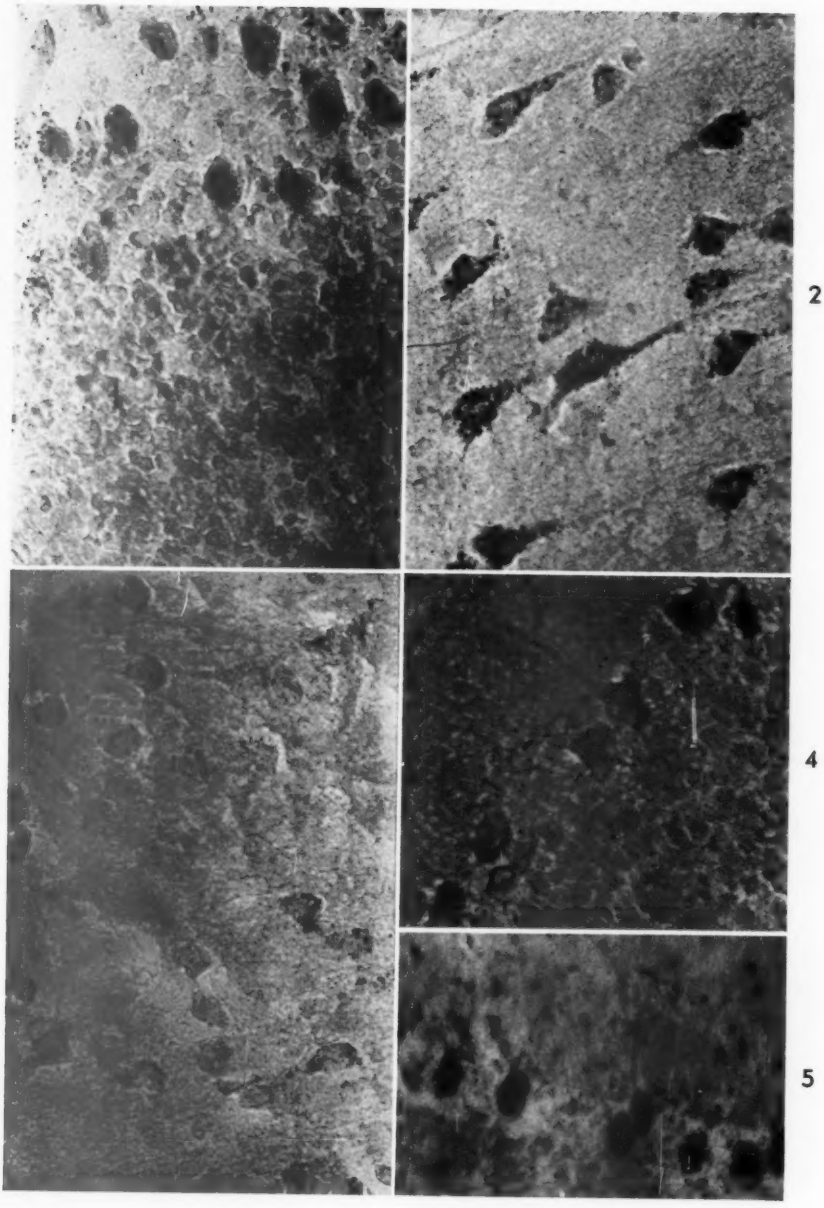
Fig. 1. Purkinje cells in the cerebellum. × 350.

Fig. 2. Large neurons in layer 5 of the neocortex. × 450.

Fig. 3. Neurons in layers 2 and 3 of the neocortex. × 450.

Fig. 4. Purkinje cells. Autolysis, 20 minutes. There is prominent enlargement and clumping of some lysosomes (arrows) with loss of the remaining normal lysosomes. × 450.

Fig. 5. Purkinje cells. Autolysis, 8 hours. There is almost complete loss of cytoplasmic activity and an increase in nuclear staining. × 250.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Figures 6 to 11 represent anoxic-ischemic lesions.

FIG. 6. Neurons in layer 4 of the neocortex of a rat killed immediately after an anoxic episode. There is swelling of the neurons with early cytolysome formation. × 700.

FIG. 7. Neurons in the pyriform cortex of the same animal shown in Figure 9. There is prominent cytolysome formation. The cytolysomes tend to be localized to the base of the apical processes. × 600.

FIG. 8. Neurons in the dorsal portion of the pyramidal cell layer of the hippocampus. Four hour survival. Many neurons are extremely shrunken or faded (arrows). Hematoxylin and eosin stain. × 300.

FIG. 9. Same region and animal shown in Figure 7. Prominent cytolysomes are present in two neurons (arrows). The remaining neurons are normal. × 500.

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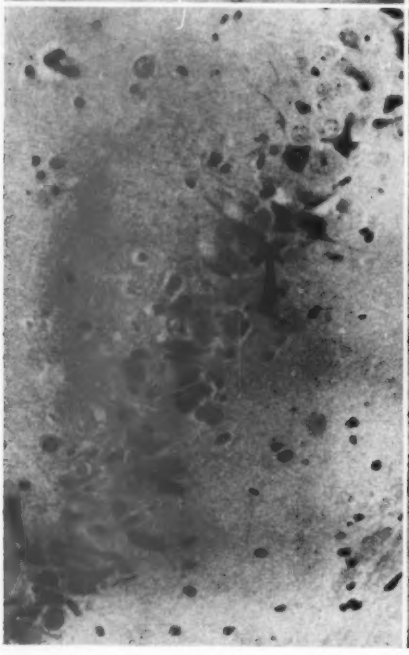

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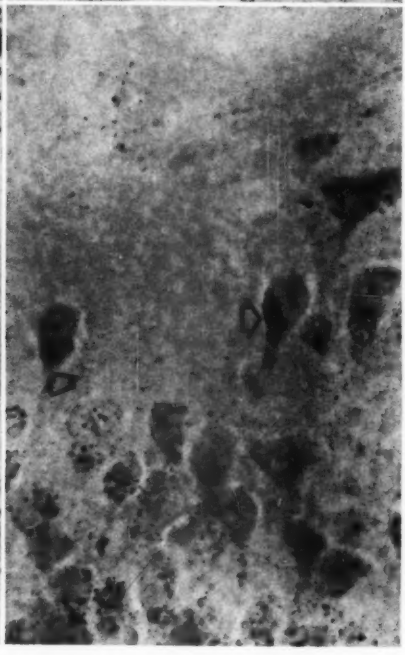

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FIG. 10. Neocortex; 24-hour survival. There is shrinkage and loss of neurons with spongy degeneration of the neuropil. Thionine stain. × 100.

FIG. 11. The same animal and infarct shown in Figure 10. Neurons with prominent cytolysomes (C) alternate with neurons which have lost most of their acid phosphatase activity (L). Substrate control sections of this tissue were negative. Note the increased nuclear and nucleolar staining. × 700.

FIG. 12. Neurons in the pyriform cortex. Animal sacrificed 4 hours after 3 episodes of acute anoxia. Most neurons contain cytolysomes (C) while a few have lost most of their enzyme activity (L). × 450.

FIG. 13. Purkinje cells. Animal killed after 3 days of chronic hypoxia. Many large cytolysomes are present. × 700.

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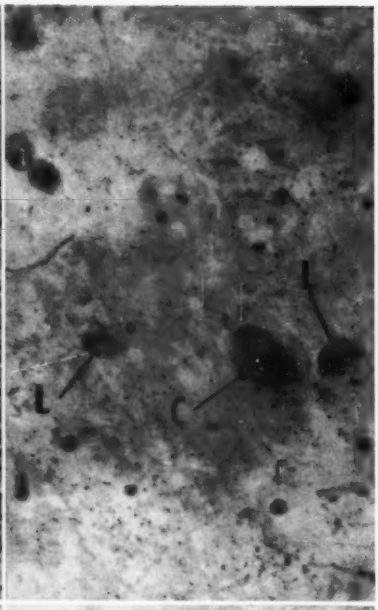

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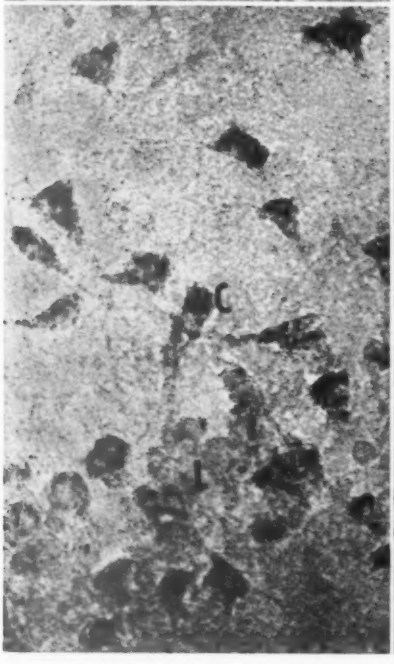

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HEALED EXPERIMENTAL GASTRIC ULCER IN THE RAT; RE-ULCERATION RESULTING FROM CORTISONE ADMINISTRATION

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There is no unanimity of opinion as to whether a peptic ulcer developing in a patient receiving cortisone therapy results from the therapy itself,[1] is coincidental,[2] or represents an adverse effect of the therapy on a pre-existing ulcer or ulcer diathesis.[3] Even the effect of cortisone administration on gastric secretory function in man is controversial. Gray, Ramsay, Reifenstein and Benson [4] have reported an increased secretory volume and acidity, while Beck, Fletcher, McKenna and Griff [5] found no significant changes in these values. The high incidence of penetration, hemorrhage, and perforation of peptic ulcers in patients receiving cortisone seems well substantiated.[6]

We have made certain observations in the rat which appear to be pertinent to this problem. Firstly,[7] it was found that thermal ulcers produced in a standard manner in the gastric fundus of the rat healed in a predictable fashion. They were all covered by regenerated mucosa within 28 days and were all completely healed by 50 days. Some of the rats were followed for periods up to 100 days, and it was found that the ulcers remained healed except for the development of small nonpenetrating mucosal erosions in the centers of the healed ulcers in some of the cases. Secondly,[8] when cortisone acetate was administered to rats immediately after the induction of gastric ulcer of this type, there were deep penetration and enlargement of the ulceration. Thirdly,[9] in a large series of rats receiving cortisone acetate for 10 months during the course of another experiment, we found no gastric erosions or ulcerations. These facts seem to indicate that cortisone has a deleterious effect on an area of active ulceration in the rat's stomach, but no effect on the intact stomach, even after prolonged administration. It seemed logical, therefore, to examine the effects of cortisone administration on rats with healed gastric ulcer.

MATERIAL AND METHODS

Male rats of the Royal Victoria Hospital strain, weighing 180 to 200 gm. at the

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time of operation, were used. They were fed Purina diet and water *ad libitum*. Ulcers were produced in the glandular portion of the stomach by a method previously described.[10] The rats were divided into the following groups:

Group I. In 69 rats an experimental gastric ulcer was produced. Fifty-five days later daily intramuscular injections of cortisone acetate (generously donated by Merck, Sharp & Dohme, Ltd., Canada), 0.05 mg. per gm. of body weight, were begun.

Group II. In 80 rats an experimental gastric ulcer was produced. Fifty-five days later daily intramuscular injections of cortisone acetate, 0.075 mg. per gm., were begun.

Group III. In 19 rats an experimental gastric ulcer was produced. Fifty-five days later a bilateral subdiaphragmatic vagotomy was performed and daily intramuscular injections of cortisone acetate, 0.05 mg. per gm., were begun.

Group IV. In 50 rats an experimental gastric ulcer was produced. No cortisone was administered.

Group V. Fifty normal rats were kept with the other experimental groups for 55 days. On day 55, daily injections of cortisone acetate, 0.075 mg. per gm., were begun.

Animals were sacrificed on the 58th, 62nd, 66th, 70th and 76th days after the induction of the ulcer. In addition, animals from Groups I, III and IV were killed at intervals from day 76 to day 100. The stomachs were fixed in formalin, and sections stained with hematoxylin and eosin.

Results

Table I summarizes the number of healed ulcers, erosions and re-ulcerations in the entire series. Table II indicates the mortality rate and the weight of the animals 76 days after induction of the ulcer.

Table I

NUMBER OF HEALED ULCERS, EROSIONS AND RE-ULCERATIONS

Group	No. in series	No. sacrificed and examined	No. with healed ulcers only	No. with erosions of a healed ulcer	No. with re-ulceration of a healed ulcer
I					
Healed ulcer, cortisone (lower dose)	69	49	37 (76%)*	5 (10%)	7 (14.3%)
II					
Healed ulcer, cortisone (higher dose)	80	61	28 (46%)	8 (13%)	25 (41%)
III					
Healed ulcer, vagotomy, cortisone (lower dose)	19	14	10 (71%)	1 (7%)	3 (21.4%)
IV					
Healed ulcer, no treatment	50	50	35 (70%)	15 (30%)	0 (0%)
V					
No ulcer, cortisone (higher dose)	50	37	0	0	0

* The percentages listed are based only on the numbers in the various groups that were actually sacrificed.

TABLE II

WEIGHT OF ANIMALS 76 DAYS AFTER ULCER INDUCTION, AND MORTALITY RATE

Group	No. in series	No. that died during experiment	Mortality rate	Wt. of animals at day 76 (gm.)
I				
Healed ulcer, cortisone (lower dose)	69	20	29%	160–190
II				
Healed ulcer, cortisone (higher dose)	80	19	23.8% *	150–170
III				
Healed ulcer, vagotomy, cortisone (lower dose)	19	5	26.3%	120–160
IV				
Healed ulcer, no treatment	50	0	0	240–260
V				
No ulcer, cortisone (higher dose)	50	13	26% *	150–180

* All of the animals in these two groups died or were sacrificed by day 76; some animals in the other groups survived to day 100.

The control rats (group IV) with healed ulcers had no mortality and gained weight steadily during the course of the experiment. The animals in the other groups gained weight normally until cortisone administration was begun. Those receiving 0.05 mg. per gm. of body weight of cortisone daily (groups I and III) lost weight for 4 or 5 days, but the weight then became stationary. The rats receiving 0.075 mg. of cortisone daily (groups II and V) lost weight during the entire period of cortisone administration. They became weak after 7 days, and those which appeared unlikely to survive were sacrificed; none of the rats in these groups survived beyond the 76th day.

The rats with experimental ulcer which were given cortisone (groups I, II and III) showed a high incidence of penetrating re-ulceration in the healed ulcer area. This was most pronounced in the group receiving the higher dose of cortisone (group II), in which 41 per cent of the rats that were killed had this type of re-ulceration, and of the animals found dead, 2 had perforated gastric ulcers and peritonitis. In the rats on the lower dose of cortisone (groups I and III), 14.3 per cent and 20 per cent, respectively, of the rats killed had penetrating re-ulceration. The animals in group III (healed ulcer, vagotomy and cortisone) had markedly dilated, thin-walled stomachs, indicating that an adequate vagotomy had been performed. Though the numbers were small, the incidence of re-

ulceration was similar to that in the group without vagotomy. No erosions or gastric ulcers were found in the rats which received cortisone but had not had an experimental ulcer induced (group V). No penetrating ulcers were found in the animals with experimental ulcer allowed to heal without the administration of cortisone (group IV) though 30 per cent of these rats did show superficial mucosal erosions.

The superficial erosions were located in the central portion of the healed ulcer and measured 1 mm. or less in diameter. The first erosion was found at day 60 in both cortisone-treated and untreated groups, and they occurred sporadically from this time onward. The penetrating re-ulcerations also occurred in the central part of healed ulcers. They measured, in most instances, at least 6 mm. in diameter. They appeared as deep, punched-out ulcers with some undermining of the edges; their floors were covered by necrotic material. In group I (lower cortisone dosage) 2 re-ulcerations were found on day 76 (21 days of cortisone) and the remainder after day 85. In group III (cortisone and vagotomy) the first re-ulceration was found on day 66 (11 days post-vagotomy and 11 days of cortisone); one appeared on day 76 and another on day 80. In group II (higher cortisone dosage) the first instance of penetrating re-ulceration was found on day 62 (7 days of cortisone), and the remainder occurred between days 66 and 76.

Microscopically, the healed ulcers in all groups had a similar appearance (Fig. 1). At the ulcer site the stomach wall was composed of dense fibrous connective tissue which replaced the normal structure of the wall. The area was covered superficially by regenerated mucosa which showed differentiation into gastric pits and glands. This lay directly on the fibrous tissue without the interposition of a *muscularis mucosae*. At 60 days and subsequently there was further shrinkage of the ulcer site due to contraction of the dense scar tissue. The healed ulcers with mucosal erosions (Fig. 2) were identical in appearance, but the superimposed erosions were small and superficial. At their deepest, the erosions involved only the full thickness of the regenerated mucosa. There was a mild acute inflammatory response bordering the erosion. In contrast, the penetrating re-ulcerations (Figs. 3 and 4) were large and deep. The base was formed by the dense connective tissue scar of the healed ulcer, but the floor was covered by a necrotic layer, beneath which was moderate hyperemia and a scattering of acute inflammatory cells. In some instances bacteria or fungi were present within the necrotic debris. Throughout the sclerotic wall of the ulcer plump fibroblasts were present, but beyond this there was no evidence of a repair process. At the ulcer margins (Fig. 5) necrosis frequently undermined the viable mucosa

which was of the same structure and degree of differentiation as the regenerated epithelium in animals with healed ulcers without re-ulceration.

Discussion

The most striking observation in this investigation was the appearance, in rats receiving cortisone, of penetrating re-ulceration in healed gastric ulcers. Rats without ulcers were given cortisone in similar dosage but did not develop ulcers, and those with healed ulcers who did not receive cortisone did not develop this type of lesion.[7] These findings suggest that cortisone was responsible for penetrating re-ulceration in healed ulcers. In a previous experiment[7] all the ulcers were completely healed by the 55th day after induction (the time when cortisone administration was begun). Thus, any penetrating ulcer found could not be due to a failure of the original experimental ulcer to heal and must have represented a new lesion. The lack of evidence of repair in the re-ulcerations suggested that the lesions were all of very recent origin. Alternatively, this would indicate a depression of the repair process by cortisone, as suggested by other observers.[11,12] The fact that re-epithelization was minimal favored a recent genesis, since epithelial proliferation has been reported to be unaffected by cortisone administration.[13] The observation of 33 extensive and deep areas of ulceration and only two small re-ulcerations also suggested that penetration took place rapidly once the process was initiated.

The results clearly indicate a relationship between dosage, time and incidence of re-ulceration. There was a 41 per cent incidence of re-ulceration in rats receiving the highest dose of cortisone, the earliest lesion appearing after 8 days of cortisone therapy. In the lower dose range there was only a 14.3 per cent incidence, and the first ulcer was found after 21 days of therapy. The mechanism by which cortisone administration results in re-ulceration is not entirely clear. We previously reported[8] that cortisone given to rats with experimental ulceration caused marked penetration, a feature which could be demonstrated within 24 hours. The evidence suggested that cortisone provoked penetration as the result of factors other than alterations in gastric secretion or depression of fibroplasia. The observations in the present experiment also suggested that the action of cortisone was not due to a modification of fibroplasia. The acute nature of the lesion and the lack of epithelial proliferation indicated rapid penetration. This would in turn lead to the hypothesis that penetration occurred before fibrous tissue proliferation could be expected to lend significant support or be of importance in limiting the extension of the lesion. It seemed unlikely that gastric hypersecretion was important. Cortisone, as administered in these ex-

periments, caused neither increased volume of gastric secretion nor increased acidity.[14] Indeed, the administration of histamine or pitressin for 30 days[15] at a dosage level which caused an increased gastric secretion and acidity in this strain of rat failed to produce re-ulceration of healed gastric ulcers. Moreover, in a group too small to draw firm conclusions, adequate vagotomy (which Jones and Harkins[16] found to reduce gastric secretion and acidity in the rat) did not prevent the occurrence of re-ulceration. From the foregoing evidence it would appear that the phenomenon of re-ulceration and penetration was not due to the action of cortisone on either gastric acidity or fibroplasia.

Cortisone could have been responsible for both the mucosal disruption and the subsequent penetration. On the other hand, the mucosal disruption could have been due to some other cause, the cortisone contributing only to the penetration. The second possibility seems to be the more likely. Kowalewski[17] reported that fasting rats receiving cortisone developed superficial gastric erosions after the fourth day; however, the rats in the present study were not fasted, and in an experiment[9] in which rats received cortisone for 10 months, no instance of gastric erosion or ulceration was encountered. In the present experiments and in others[7] mucosal erosions occurred spontaneously in healed experimental gastric ulcers. In the absence of cortisone administration, penetration did not occur, and the erosions seemed to heal readily. It is possible that with the administration of cortisone the spontaneous erosions failed to heal and progressed to penetration. There appears to be some evidence to suggest that the main action of cortisone in this experiment was to cause penetration of mucosal erosions in healed gastric ulcers.

Although in man the ulcerogenic action of cortisone has recently been challenged,[2] there is no doubt that in rats cortisone administration can result in re-ulceration of healed gastric ulcers. These, with their rapidity of penetration, bear some resemblance to the re-activation of healed peptic ulcers in patients receiving cortisone. In these instances the rapidity of penetration probably accounts for the high incidence of such complications as hemorrhage and perforation.

Summary

Experimental gastric ulcers were produced in a group of rats by means of thermocautery. Fifty-five days after production of the ulcers, by which time they were completely healed, intramuscular injections of cortisone acetate were begun. One group received 0.05 mg. per gm. of body weight daily and a second group, 0.075 mg. daily. Both groups receiving cortisone showed a high incidence of penetrating re-ulceration in the healed ulcer area. This was more pronounced in the group re-

ceiving the higher dose (41 per cent) than in the group given the lower dose of cortisone (14.3 per cent). In contrast, a group of rats with a similar healed ulcer, but not receiving cortisone, had no re-ulceration. The mechanism by which cortisone results in penetrating re-ulceration of a healed gastric ulcer in the rat was not fully elucidated by this investigation, but it appeared unlikely to be due to the action of cortisone on either gastric acidity or fibroplasia. The results of the experiment bore some resemblance to the re-activation of healed peptic ulcer in patients receiving cortisone.

References

1. Hall, B. D. Gastric ulcer associated with cortisone therapy. *Gastroenterology,* 1953, **25**, 80–83.
2. Palmer, W. L., and Kirsner, J. B. Therapeutic and side effects of the anti-inflammatory steroids on the gastrointestinal tract. *Ann. New York Acad. Sc.,* 1959, **82**, 947–956.
3. Smyth, G. A. Activation of peptic ulcer during pituitary adrenocorticotropic hormone therapy; report of 3 cases. *J.A.M.A.,* 1951, **145**, 474–477.
4. Gray, S. J.; Ramsay, C.; Reifenstein, R. W., and Benson, J. A., Jr. The significance of hormonal factors in the pathogenesis of peptic ulcer. *Gastroenterology,* 1953, **25**, 156–172.
5. Beck, I. T.; Fletcher, H. W.; McKenna, R. D., and Griff, H. Effect of small and massive doses of prednisone on gastric secretory activity. *Gastroenterology,* 1960, **38**, 740–749.
6. Bergentz, S. E. Ulceration of the stomach attending cortisone and corticotropin therapy; report of a case. *Acta chir. scandinav.,* 1955, **109**, 334–338.
7. Phillips, M. J.; Skoryna, S. C., and Kahn, D. S. Studies on the healing of experimental penetrating gastric ulcer in the rat. *Canad. J. Surg.,* 1961, **4**. (In press.)
8. Kahn, D. S.; Phillips, M. J., and Skoryna, S. C. The early penetration of experimental gastric ulcers in rats receiving cortisone. *Am. J. Path.,* 1961, **38**, 73–87.
9. Kahn, D. S., and Skoryna, S. C. Effects of long-term cortisone administration on the skeletal tissue of the rat and on the bone tumors produced by radioactive strontium. *Lab. Invest.,* 1959, **8**, 763–776.
10. Skoryna, S. C.; Webster, D. R., and Kahn, D. S. A new method of production of experimental gastric ulcer; the effects of hormonal factors on healing. *Gastroenterology,* 1958, **34**, 1–10.
11. Ragan, C.; Howes, E. L.; Plotz, C. M.; Meyer, K., and Blunt, J. W. Effect of cortisone on production of granulation tissue in the rabbit. *Proc. Soc. Exper. Biol. & Med.,* 1949, **72**, 718–721.
12. Spain, D. M.; Molomut, N., and Haber, A. The effect of cortisone on the formation of granulation tissue in mice. (Abstract) *Am. J. Path.,* 1950, **26**, 710.
13. Williams, A. W. Influence of cortisone on the healing of gastric ulcers. *J. Path. & Bact.,* 1954, **67**, 259–261.
14. Jowd, E., and Skoryna, S. C. Effect of cortisone administration on gastric secretion in the rat. (In preparation.)

15. SKORYNA, S. C.; PHILLIPS, M. J.; JOWD, E.; WEBSTER, D. R., and KAHN, D. S. The effect of histamine and pitressin administration on gastric secretory function and on experimental gastric ulcers in the rat. (In preparation.)

16. JONES, T. W., and HARKINS, H. N. Evaluation of mechanisms involved in gastric acid secretion of pylorus-ligated rats. *Gastroenterology*, 1958, **35**, 309–311.

17. KOWALEWSKI, K. Effect of certain androgenic steroids and cortisone on gastric ulcerogenesis in fasting rats. *Proc. Soc. Exper. Biol. & Med.*, 1959, **101**, 147–150.

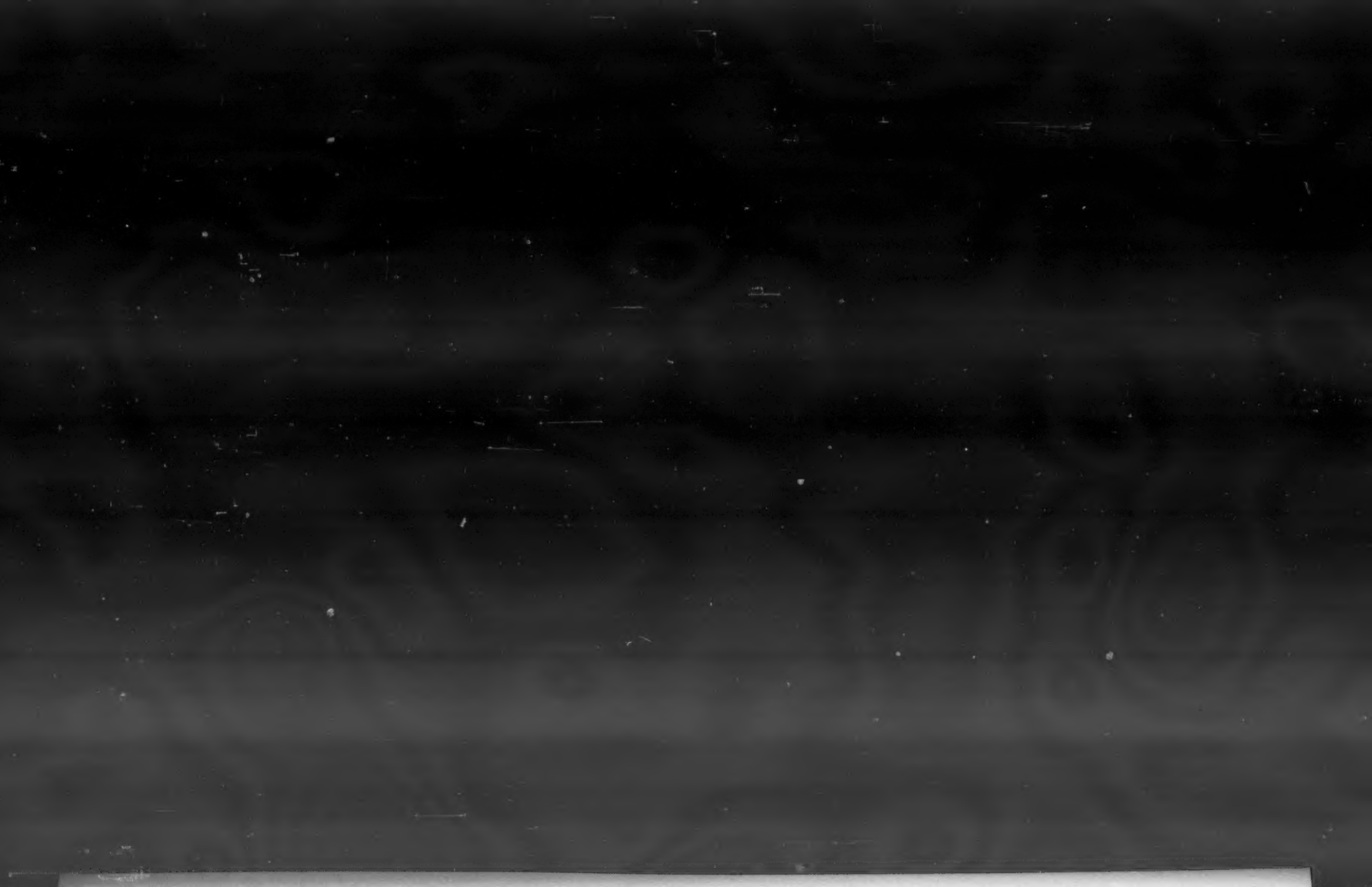
LEGENDS FOR FIGURES

Illustrations were prepared from sections stained with hematoxylin and eosin.

FIG. 1. A healed gastric ulcer 55 days after its induction and before cortisone administration was begun. The edges of the intact muscle coat at the margin of the healed ulcer can be seen at M. The wall of the stomach between these two edges is composed of connective tissue which has filled in the original ulcer area. The mucosal surface of the healed ulcer consists of regenerated epithelium which lies directly on the fibrous tissue wall without the interposition of a *muscularis mucosae*. × 20.

FIG. 2. A healed gastric ulcer 76 days after its induction (after 21 days of cortisone, 0.075 mg. per gm. of body weight daily). A small superficial erosion can be seen in the central portion of the healed ulcer. × 60.

FIG. 3. Penetrating re-ulceration of a healed gastric ulcer 76 days after induction of the ulcer (after 21 days of cortisone, 0.05 mg. per gm. of body weight daily). Normal stomach wall is seen peripherally at M. Penetration into the fibrous tissue wall of the healed ulcer and marginal undermining can be seen. × 20.



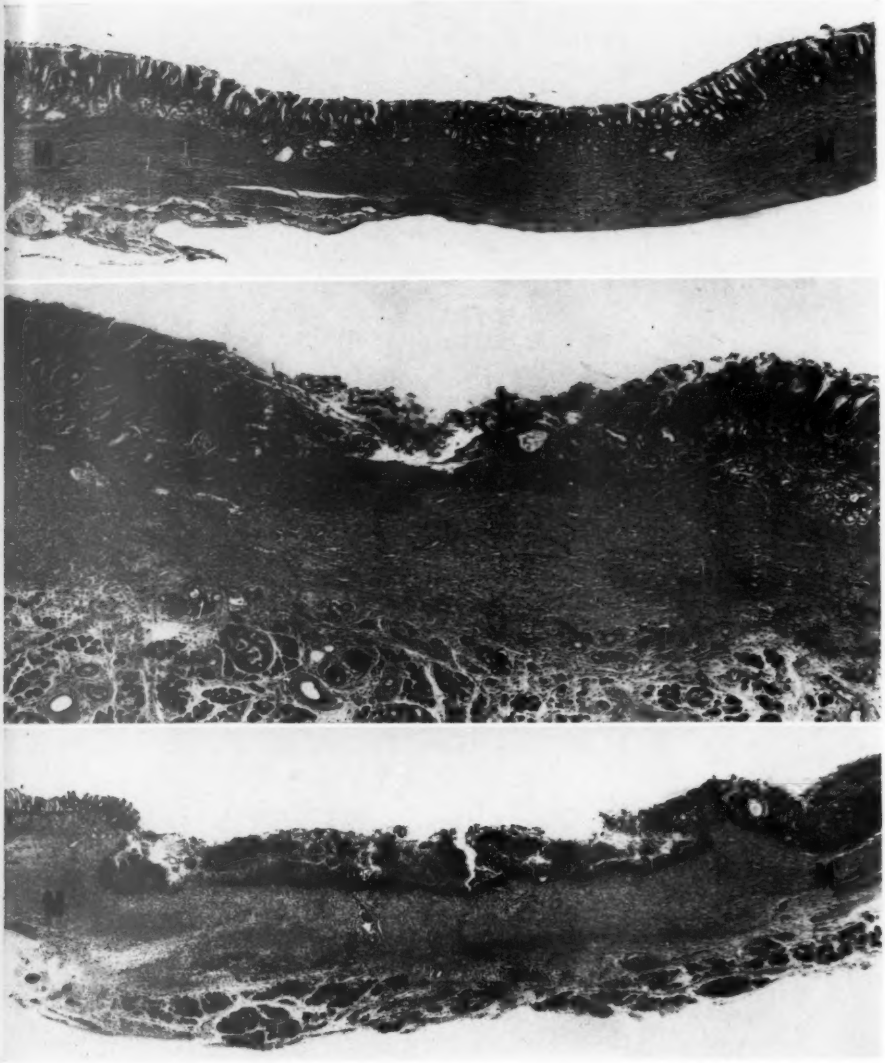


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FIG. 4. Penetrating re-ulceration of a healed gastric ulcer 62 days after induction of the ulcer (after 7 days of cortisone, 0.075 mg. per gm. daily). The deep penetration and the mucosal undermining into the intact muscle (M) at the periphery are manifest. × 40.

FIG. 5. High power view of the intact muscle (M) in Figure 4. The undermining into the intact muscle at the margin of the healed ulcer is shown. × 100.

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5

STUDIES ON THE ADJUVANT ACTION OF BACTERIAL ENDOTOXINS ON ANTIBODY FORMATION

IV. Histologic Study of Cortisone-Treated Rabbits

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The ability of adrenal cortical hormones to suppress both the primary and the secondary antibody response to soluble and insoluble antigens is well documented.[1] Associated with this inhibition is a differential susceptibility of cells of lymphoid tissue to the destructive effects of cortisone.[2] An accelerated destruction of mature lymphocytes occurs, which is brief and characterized by pyknosis, karyorrhexis, and cytoplasmic shedding. This is followed by a prolonged phase of diminished production of lymphocytes. The mature lymphocytes and plasma cells have been found to be quite susceptible to cortisone whereas the reticuloendothelial cells and immature lymphocytes (known as "reticular lymphocytes," according to the terminology of Sundberg and Downey[3]) undergo minimal alteration.

In view of the emphasis placed on the role of plasma cells and lymphocytes in antibody production, it is possible that cortisone might inhibit the immune response by causing destruction of these cell types. However, the actual synthesis of antibody is probably associated with undifferentiated immature blast cells, the ultimate end product of which may vary under different experimental conditions.[4] From a morphologic standpoint, these undifferentiated cells do not appear to be destroyed by cortisone, as evidenced in a histologic study by Craig[5] of popliteal lymph nodes from cortisone-treated rabbits given an injection of *Salmonella typhosa* O antigen in the homolateral foot pad. With the schedule of cortisone treatment used, mature lymphocytes were destroyed, but antibody titers were not appreciably affected. The response described by Craig, although delayed approximately 4 days by cortisone, consisted of marked follicular activity in lymph nodes with the formation of large secondary nodules (germinal centers). These areas had considerable mitotic activity as well as intense pyroninophilia. Similar changes were noted in the medullary cords. A reticular or transitional cell was the active cell type in both areas, and was linked to antibody production.

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In a previous publication [6] we have described the histologic changes in spleens of rabbits following a single injection of bovine gamma globulin (BGG) alone and in combination with an adjuvant (bacterial endotoxin). Antibody synthesis as well as its enhancement by endotoxin was correlated with increased mitotic activity and differentiation of primitive reticular cells in the splenic follicles. This cell, referred to as a modified reticular cell, appeared to be similar to that described by Craig,[5] and implicated earlier by Fagraeus,[7] Harris and Harris,[8] and other investigators [9,10] in the synthesis of antibody. It was also established that the potent adjuvant action of endotoxins derived from gram-negative bacteria could stimulate the synthesis of antibody to human serum albumin in rabbits given inhibitory doses of cortisone.[11] The present investigation was carried out to ascertain the location and cell type of the lesions in spleens of cortisone-treated rabbits following antigen injection with and without stimulation by endotoxins. Antibody titers were found once again to correlate closely with the appearance and activity of pyroninophilic reticular cells in splenic follicles.

Material and Methods

White male New Zealand rabbits, 6 months old, weighing 2 to 3 kg., were used. Cortisone (Cortone Acetate,® Merck, Sharp and Dohme) was injected daily into the gluteal areas of the rabbits, alternating the right and left sides, in a constant dose of 10 mg. (0.1 ml.). After 5 days of cortisone therapy, approximately 5 mg. of potassium chloride were added daily to the drinking water of each animal. The antigen, bovine gamma globulin, fraction II, Armour and Company, was diluted to a concentration of 10 mg. per ml. in phosphate-buffered saline, pH 7.2, and 1 ml. volume injected intravenously into the marginal vein of the ear. The endotoxin was derived from *Escherichia coli* strain 0:111 B4 by the procedure of Boivin and Mesrobeanu [12] and furnished by Difco Company, Detroit, Michigan. It was diluted similarly to concentrations permitting the injection of a constant total volume of 1 ml. of various products.

Plan of Experiment

Three groups of 21 rabbits each were used in the experiment. The products they received and the group designations were as follows: group E received 10 mg. of bovine gamma globulin with 5 μg. of endotoxin, plus cortisone treatment from 5 days before antigen administration through the 12 days following (-5 to $+12$ days); group F received 10 mg. of bovine gamma globulin, plus cortisone -5 to $+12$ days; group G received cortisone -5 to $+12$ days.

Following intravenous injection of the appropriate products, animals were sacrificed in lots of 3 by cardiac exsanguination 1, 2, 4, 6, 8, 12, and 18 days following injection of antigen. Blocks of tissue from the spleen, thymus gland, liver, and periportal lymph nodes were removed and placed in Carnoy's fixative and 10 per cent neutral formalin. Following the techniques used in the previous investigation,[6] histologic sections were prepared utilizing hematoxylin and eosin stain and a modified Unna-Pappenheim stain.

Titrations for antibody were done according to a hemagglutination technique,[13] with serums from all groups tested at the same time.

Results

Antibody Titrations

Hemagglutinin titers of cortisone-treated animals receiving bovine gamma globulin (BGG) with and without endotoxin are given in Table I. Antibody in the group receiving BGG alone (group F) was usually present in only trace amounts. Six days after cortisone therapy had ceased (on the 18th day) there was no serologic evidence of a rebound phenomenon. In contrast, animals which received endotoxin with antigen (group E) had antibody levels considerably higher than the group not receiving endotoxin. Of interest is the fact that antibody titers in group E were also high on the 18th day, with 2 of 3 animals exhibiting titers of 1:5,120. As will be discussed below, maximum serologic activity coincided in general with maximum histologic changes.

Table I

COMPARATIVE ANTIBODY TITERS OF ENDOTOXIN AND NON-ENDOTOXIN TREATED RABBITS RECEIVING CORTISONE

Rabbit no.	Days following injection	Reciprocal of hemagglutinating titer	
		BGG alone (group F)	BGG + endotoxin (group E)
4		—*	—
5	1	—	—
6		—	—
7		—	—
8	2	—	—
9		—	—
10		—	—
11	4	—	—
12		—	—
13		—	1280
14	6	—	5120
15		20	1280
16		640	5120
17	8	40	640
18		—	640
19		80	640
20	12	20	640
21		160	640
22		20	5120
23	18	10	5120
24		not done	640

* Minus sign indicates a titer less than 1:10.

Histologic Alterations

General. After 10 days of cortisone treatment, the lymph nodes and thymic glands from animals of all 3 groups were very small, and the

parenchymatous content was markedly reduced. The differences in these tissues among the 3 groups were minimal. The gross sizes of spleens in all groups were found to be smaller than normal, with weights being variable. However, histologic alterations which could be correlated with an antibody response were confined to the splenic tissue in group E.

Group G (cortisone from −5 to +12 days; no bovine gamma globulin or endotoxin). After 7 to 10 days of cortisone treatment, (i.e. comparable to the first 4 days following antigen stimulation in groups E and F) the red pulp and the follicles of the spleen were diffusely infiltrated with heterophils. The lymphocytic cells of the mantle layers of the follicles were greatly reduced in number. The follicles consisted essentially of areas of pale staining cells (hematoxylin and eosin stain), with central collections of reticuloendothelial elements that merged almost imperceptibly with wide marginal zones of monocytic cells. Although the follicles were about ½ to ⅓ the size of those in normal uninjected rabbits, it appeared that the central reticuloendothelial and the marginal monocytic cells were present in normal numbers. The striking change was the disappearance of the mature, medium-sized follicular lymphocytes. Occasional small foci of hyalin were discernible in scattered follicles.

By the sixth day (the twelfth day of cortisone treatment), the infiltration of both red and white pulp by heterophils had virtually disappeared, and the red pulp appeared normal. The follicles were almost devoid of mature lymphocytes but again contained normal numbers of central reticuloendothelial and marginal monocytic cells; they remained small, with a diameter of ¼ to ⅙ the normal size. The tissues examined on the eighth and twelfth days were similar in appearance. Mitotic figures (which usually appeared in numbers of 2 to 3 per high power field in about one-half the follicles of normal controls) were rarely seen. In one specimen (G 20) all follicular architecture was lost, being replaced by monocytic and spindle cells. On the 18th day, or 6 days after the cessation of cortisone treatment, the follicles were partially reconstituted, but still less than normal size (Figs. 3 and 4).* Medium-sized, mature lymphocytes were present in about one-half the normal number. A few mitotic figures were present in the central reticular areas but were never as frequent as in the normal controls. The red pulp appeared normal.

Group F (cortisone, −5 to +12 days; bovine gamma globulin, 10 mg. on day 0). The changes during the first 8 days were similar to those in

* The histologic changes in spleens of all animals of these experiments were carefully compared with those previously observed.[6] Figures 1 and 2, demonstrating tissues in a normal control animal, and the reaction produced by the combined administration of endotoxin and bovine gamma globulin, are included for purposes of comparison.

group G. The follicular lymphocytes had disappeared almost completely, while the reticuloendothelial elements of the follicles were present in normal numbers. Scattered foci of hyalin were seen within follicles. On the twelfth day (last day of cortisone treatment) 2 specimens exhibited alterations similar to the 6 and 8 day samples. A third spleen (F 19) contained follicles with small, discrete germinal centers of modified reticular cells (large immature reticular cells with a rim of pyroninophilic cytoplasm). Mitotic figures were as frequent as 5 per high power field. However, these modified reticular cells were very loosely packed, unlike the tightly packed areas described previously.[6] All specimens on the twelfth day had an amount of white pulp which was less than one-half that of normal non-cortisone-treated controls. On the 18th day the white pulp remained reduced (Fig. 5); 2 to 3 mitotic figures were present in central follicular areas, but there were no significant aggregations of modified reticular cells (Fig. 9). However, greater numbers of mature lymphocytes were present in the follicles than noted previously. The morphologic findings in group F did not suggest any significant antibody response.

Group E (cortisone, −5 to +12 days; bovine gamma globulin, 10 mg., and endotoxin, 5 μg. on day 0). For the first 2 days following the injection of antigen, the findings were similar to those previously described for groups F and G, consisting of a diffuse infiltration by heterophils and a marked numerical reduction of mature follicular lymphocytes. The reticuloendothelial and monocytic elements of the follicles were present in normal numbers. On the fourth day, the findings in 2 specimens were similar to those of the first 2 days (Figs. 6 and 11); one (E 10) revealed a complete loss of the normal follicular architecture. A third spleen (E 12) had small distinct central areas within the follicles with an increase in the number of modified reticular cells and 4 to 5 mitotic figures per high power field. However, no antibody was detectable in the serum. On the sixth day, antibody levels became prominent, and in the spleens of 2 rabbits small discrete aggregates of modified reticular cells with increased mitotic activity (5 to 10 mitotic figures per high power field) could be seen in the follicles. One rabbit (E 15) also possessed significant levels of antibody, but only changes similar to those in groups F and G were visible in the spleen at this time. The follicular lymphocytes remained reduced in number. On the eighth day, the spleen from rabbit E 18 had small centers of modified reticular cells and mitotic figures numbered 2 to 5 per high power field, while the spleen of rabbit E 17 was similar to those described in groups F and G. A third specimen (E 16; antibody titer, 1:5,120) revealed more pronounced alterations with 10 mitotic figures per high power field. In this animal, the overall follic-

ular size was about normal, although the central reticular area accounted for more than one-half the total size.

The splenic follicles of all 3 rabbit spleens on the twelfth day contained small, central aggregations of densely packed modified reticular cells. In one specimen mitotic activity was present (5 to 10 figures per high power field), but it had diminished in the other two. Antibody levels were relatively low, 1:640 in all rabbits. Small aggregates of pyroninophilic cells were seen occasionally in the red pulp. The white pulp remained depleted of lymphocytes. On the 18th day the splenic follicles in 2 of 3 animals were maximally enlarged, being 3 to 5 times larger than any observed during the first 12 days (Figs. 7, 8 and 10). This coincided with an increase in serum antibody titers which reached a level approximately 8 times that observed on the twelfth day. The increase in follicular size could be attributed mainly to densely packed, modified reticular cells (Fig. 12). Mitotic figures were present in greatest numbers (20 per high power field) at this time also. The follicles still exhibited a significant loss of mature lymphocytes, sometimes making it difficult to determine the boundary between the reticular cell mass centrally, and the surrounding mantle layer. A third specimen (E 24) showed follicles with small discrete aggregates of modified reticular cells, but with minimal mitotic activity (2 to 5 figures per high power field). The antibody titer of this animal was approximately ⅛ of the preceding two. The red pulp appeared normal with no significant numbers of plasma cells being observed.

Discussion

The primary antibody response incited by the adjuvant action of endotoxin in cortisone-treated rabbits was associated with the appearance and proliferation of modified reticular cells (pyroninophilic) in the centers of splenic follicles. Data consistent with the hypothesis that changes in the germinal centers of follicles in the lymph nodes and spleen are associated with the production of antibody have been obtained repeatedly.[14] Further substantiation is offered in this study and by our recent report that a marked increase in size of and activity within splenic follicles were the only morphologic alterations which could be correlated with the profound increase in primary antibody levels observed when the endotoxin adjuvant is incorporated with antigen.[6] In addition, an active role of the white pulp in antibody formation is indicated from the findings of Ortega and Mellors [15] that the splenic follicles of man are a source of gamma globulin.

The histologic features described herein consisted of variable degrees of activity on the sixth and eighth days within splenic follicles of rabbits

in group E, with a tendency to wane on the twelfth day after antigenic stimulus. Following this, follicular activity and serum antibody levels were found to be markedly increased on the 18th day, 6 days after cessation of cortisone treatment. This burst in both activities after cortisone withdrawal supports the concept that the increased follicular response is related to the synthesis of antibody. In addition, the number of mitotic figures and the degree of pyroninophilia within splenic follicles, in general, correlated with the level of serum antibody. On the other hand, except for a single instance (F 16), only trace amounts of hemagglutinating antibody were observed in cortisone-treated animals receiving antigen in the absence of endotoxin. In such inhibited animals, the splenic follicular response was absent. No serologic or histologic "rebound" phenomenon was observed 6 days after cortisone was discontinued (day 18). Partial reconstitution of the follicles with mature, medium-sized lymphocytes, however, had occurred at this time.

The mechanism by which cortisone inhibits antibody synthesis is not known. However, there was no apparent morphologic evidence of damage to the reticuloendothelial cells of the splenic follicles in any of the 3 groups of cortisone-treated animals. Yet, circulating antibody and the follicular response, consisting of proliferation and differentiation of primitive reticular cells, were prominent only when endotoxin was given in combination with antigen. The fact that little or no antibody appeared in the cortisone-treated rabbits receiving only the antigen, despite the apparent morphologic normality of these cells, implies that cortisone inhibition of antibody production takes place by means other than by detectable structural cellular damage. In these animals inhibition may be more likely attributed to effects on antigen metabolism within the cell. At any rate, it was apparent that the undifferentiated reticular cells did not react to and become sensitized to the antigen.

An additional property of cortisone, that of inhibition of mitosis, may also play a role in suppressing the antibody response. For example, evidence was previously presented[11] that cortisone treatment during primary antigenic stimulus prevented an animal from responding in a characteristic earlier and rapid manner to a second injection of the antigen, human serum albumin. This suggested that cortisone inhibition could occur by preventing initial conditioning of the antibody forming mechanism, or by preventing the increase in numbers of conditioned cells. The necessity of the latter for antibody formation has been discussed by LaVia, Uriu and Ferguson.[16] While it is difficult to differentiate between these two hypothetical factors, inasmuch as the former is probably required for the latter, our observations during this study indicate that even though sensitization was induced in an unknown manner by endo-

toxin, cortisone may have exerted some deleterious effect on the mitotic activity of these cells. Thus, this hormone seemed to suppress the intense mitotic activity associated with the production of elevated levels of antibody by the endotoxin adjuvant in the previous study.[6] Although in animals given endotoxin with BGG, conditioning does occur, inasmuch as antibody titers were readily measurable and cellular proliferation was increased over that of normal control animals, it was not until cortisone treatment had ceased that mitotic activity reached a maximum (day 18). The suppression of mitotic activity during treatment with cortisone was also noted by Craig.[5]

The fact that antibody production (under endotoxin stimulus) proceeded in the virtual absence of mature lymphocytes tends to negate the importance of this cell *per se,* as being significant in the production of antibody under our experimental conditions. A similar conclusion was reached by Craig.[5] As in our previous study,[6] any role played by plasma cells and the red pulp of the spleen under conditions representative of the primary response in rabbits, was not apparent.

Summary

A morphologic investigation of the primary antibody response to bovine gamma globulin stimulated by a bacterial endotoxin in cortisone-treated rabbits has been described. Antibody production was associated with the appearance and proliferation of pyroninophilic reticular cells in the centers of splenic follicles.

References

1. Kass, E. H., and Finland, M. Adrenocortical hormones in infection and immunity. *Ann. Rev. Microbiol.*, 1953, **7**, 361–388.
2. Dougherty, T. F. Some observations on mechanisms of corticosteroid action on inflammation and immunologic processes. *Ann. New York Acad. Sc.*, 1953, **56**, 748–756.
3. Sundberg, R. D., and Downey, H. Comparison of lymphoid cells of bone marrow with lymph nodes of rabbits and guinea pigs. *Am. J. Anat.*, 1942, **70**, 455–499.
4. Fagraeus, A. Cellular reaction in antibody formation. *Acta haemat.*, 1958, **20**, 1–8.
5. Craig, J. M. The histology of antigenically stimulated lymph nodes in rabbits given ACTH or cortisone. *Am. J. Path.*, 1952, **28**, 629–651.
6. Ward, P. A.; Johnson, A. G., and Abell, M. R. Studies on the adjuvant action of bacterial endotoxins on antibody formation. III. Histologic response of the rabbit spleen to a single injection of a purified protein antigen. *J. Exper. Med.*, 1959, **109**, 463–474.
7. Fagraeus, A. Antibody production in relation to the development of plasma cells. *Acta med. scandinav.*, 1948, suppl. **204**, 122 pp.
8. Harris, T. N., and Harris, S. Histochemical changes in lymphocytes during

the production of antibodies in lymph nodes of rabbits. *J. Exper. Med.*, 1949, 90, 169–180.

9. LEDUC, E. H.; COONS, A. H., and CONNOLLY, J. M. Studies on antibody production. II. The primary and secondary responses in the popliteal lymph node of the rabbit. *J. Exper. Med.*, 1955, **102**, 61–72.
10. GOOD, R. A. Morphological Basis of the Immune Response and Hypersensitivity. In: Host-Parasite Relationships in Living Cells. FELTON, H. M. (ed.) Charles C Thomas, Springfield, Ill., 1957, pp. 78–160.
11. WARD, P. A., and JOHNSON, A. G. Studies on the adjuvant action of bacterial endotoxins on antibody formation. II. Antibody formation in cortisone-treated rabbits. *J. Immunol.*, 1959, **82**, 428–433.
12. BOIVIN, A., and MESROBEANU, L. Recherches sur les antigènes somatiques du bacille typhique. Sur la nature chimique des antigènes "O" et "Vi." *Compt. rend. Soc. de biol.*, 1938, **128**, 5–8.
13. LANDY, M., and TRAPANI, R. J. A hemagglutination test for plague antibody with purified capsular antigen of Pasteurella pestis. *Am. J. Hyg.*, 1954, **59**, 150–156.
14. HARRIS, T. N., and HARRIS, S. The genesis of antibodies. *Am. J. Med.*, 1956, 20, 114–132.
15. ORTEGA, L. G., and MELLORS, R. C. Cellular sites of formation of gamma globulin. *J. Exper. Med.*, 1957, **106**, 627–640.
16. LAVIA, M. F.; URIU, S. A., and FERGUSON, L. A. Studies on antibody production by spleen explants maintained *in vitro*. *J. Immunol.*, 1960, **84**, 48–53.

[*Illustrations follow*]

Legends for Figures

All photographs were prepared from sections stained with hematoxylin and eosin.

Fig. 1. Spleen from a normal control rabbit. A representative lymphoid follicle consists of a small, pale, inactive center of reticuloendothelial cells, surrounded by a dark mantle zone of small lymphocytes and a pale marginal zone of monocytic cells. × 140.

Fig. 2. Spleen of a rabbit, 8 days after the administration of 10 mg. of bovine gamma globulin (BGG) and 5 μg. of endotoxin. A representative follicle contains a large central area of pale, modified, reticular cells, bordered by a dark zone of mature lymphocytes. This section was taken from an animal in a previous experiment and is included for purposes of comparison. × 140.

Fig. 3. Spleen from a rabbit in group G, 18 days after the administration of cortisone from days −5 to +12; no antigen. There is a striking reduction in follicular substance, and the central arterioles have little surrounding white pulp. × 90.

Fig. 4. This section is from the same spleen depicted in Figure 3. Follicular substance is markedly reduced, particularly the reticuloendothelial center and the mantle zone of lymphocytes. × 140.

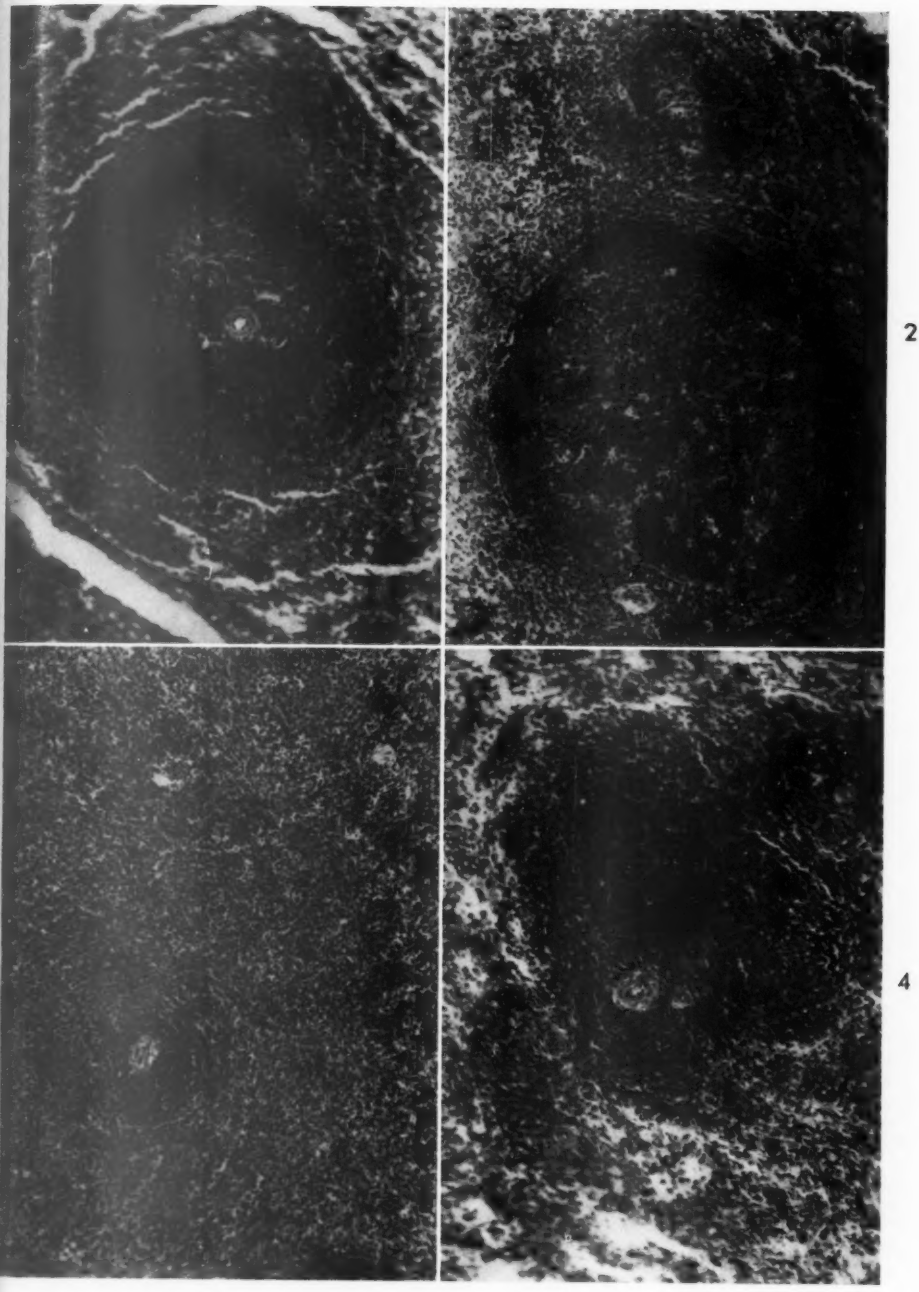
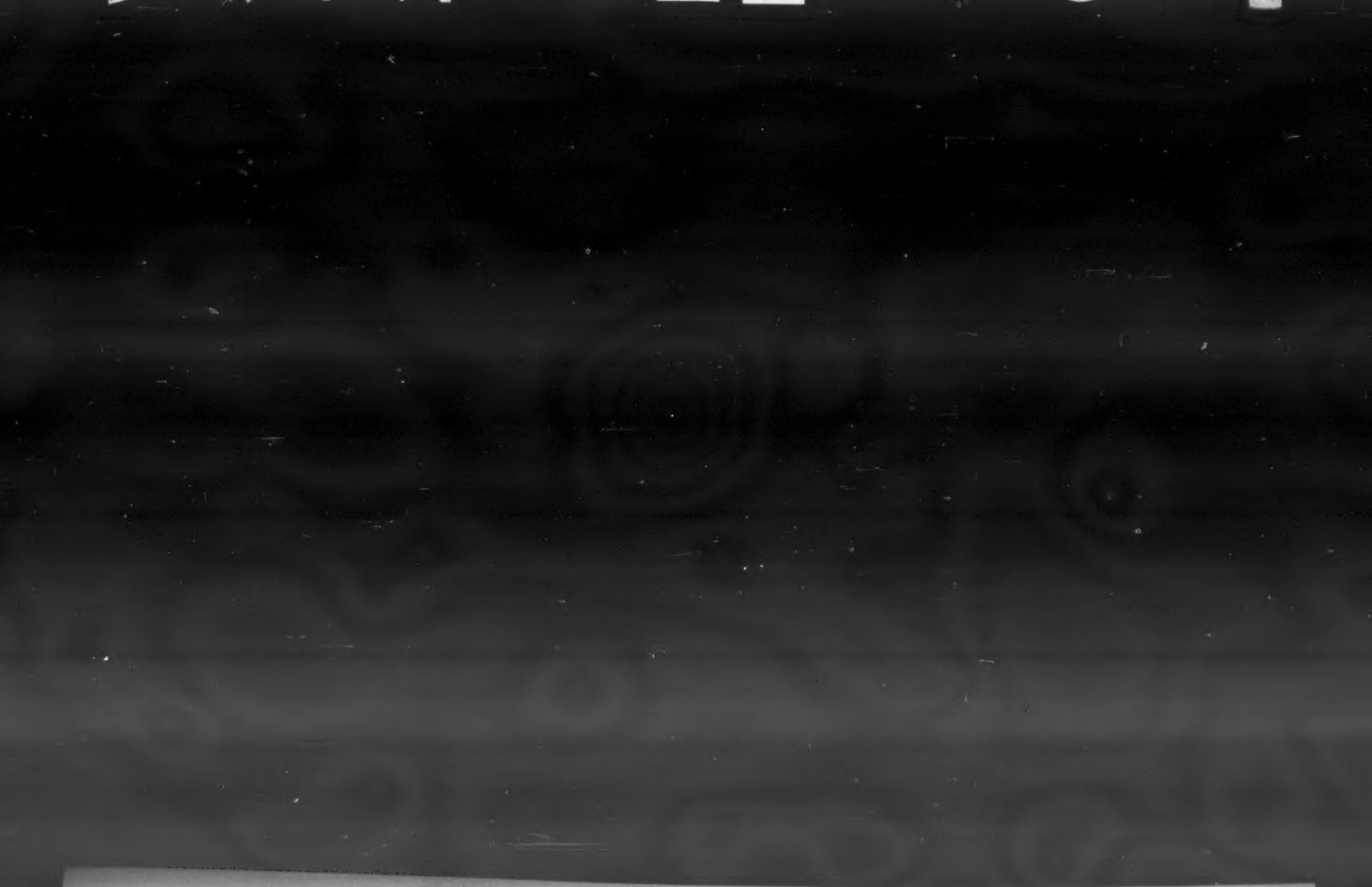


FIG. 5. Spleen from a rabbit in group F, 18 days after the administration of cortisone from days −5 to +12 and 10 mg. of BGG on day 0. The follicle is approximately the size of those in spleens of normal uninjected controls, but the reticular center is ill-defined and lymphocytes are reduced in number. The change is similar to that observed after the administration of cortisone and no antigen. × 140.

FIG. 6. Spleen from a rabbit in group E, 4 days after the administration of cortisone from days −5 to +12 and 10 mg. of BGG with 5 μg. of endotoxin. The follicle is small, nonreactive, and depleted of small dark-staining lymphocytes. Consequently, it is difficult to distinguish the peripheral zone of monocytes from the central reticular cells which are present in normal numbers. × 140.

FIG. 7. Spleen from a rabbit in group E, 18 days after the administration of cortisone from days −5 to +12 and 10 mg. of BGG with 5 μg. of endotoxin on day 0. The follicles are prominent, being 3 to 5 times larger than in normal uninjected controls. The central area of modified reticular cells comprises the largest component of the white pulp. The dark intermediate zone of lymphocytes is only partially reconstituted. Antibody titers were at peak levels at this time. × 90.

FIG. 8. This section is from the same spleen depicted in Figure 7. The lymphoid follicle is much larger than normal, and there is a marked proliferation of central reticuloendothelial elements. × 140.



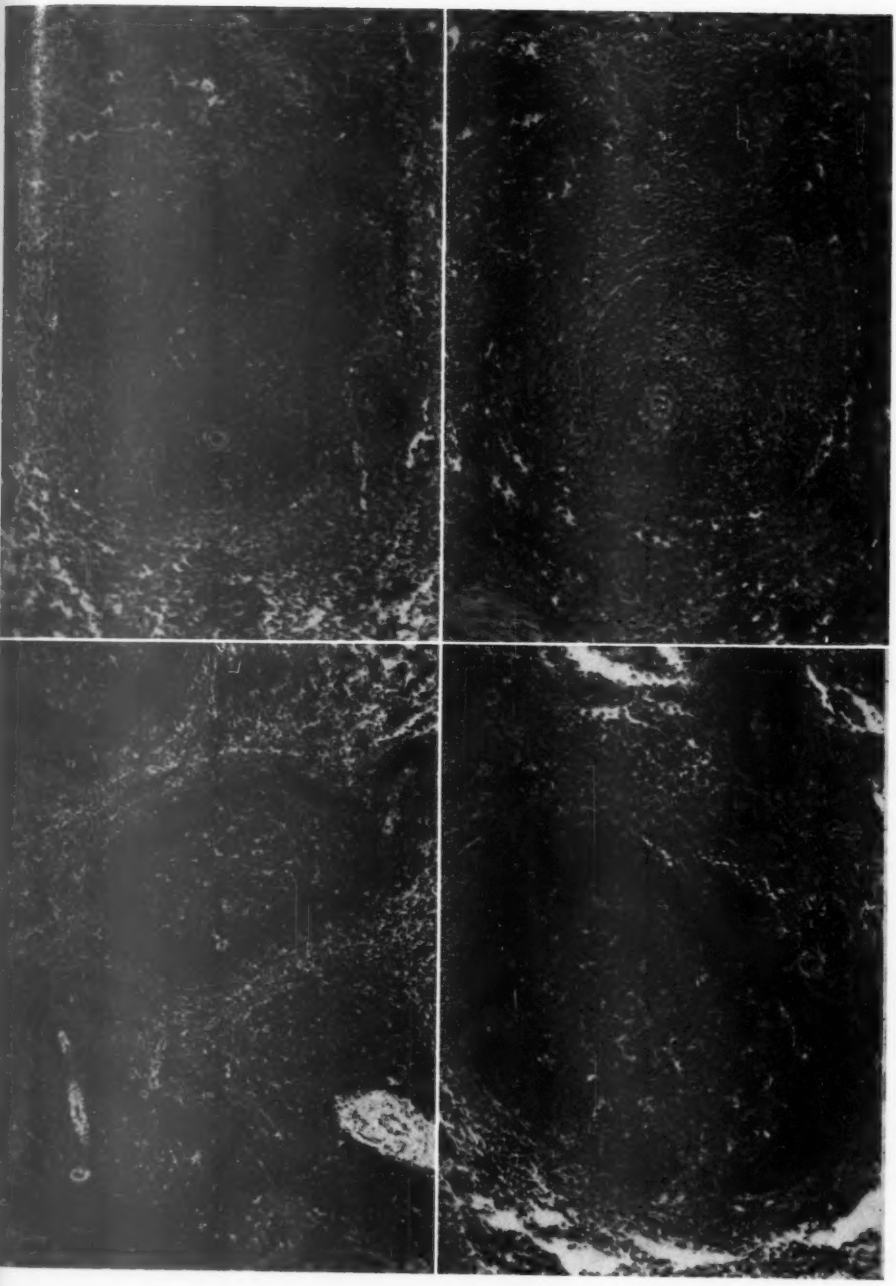
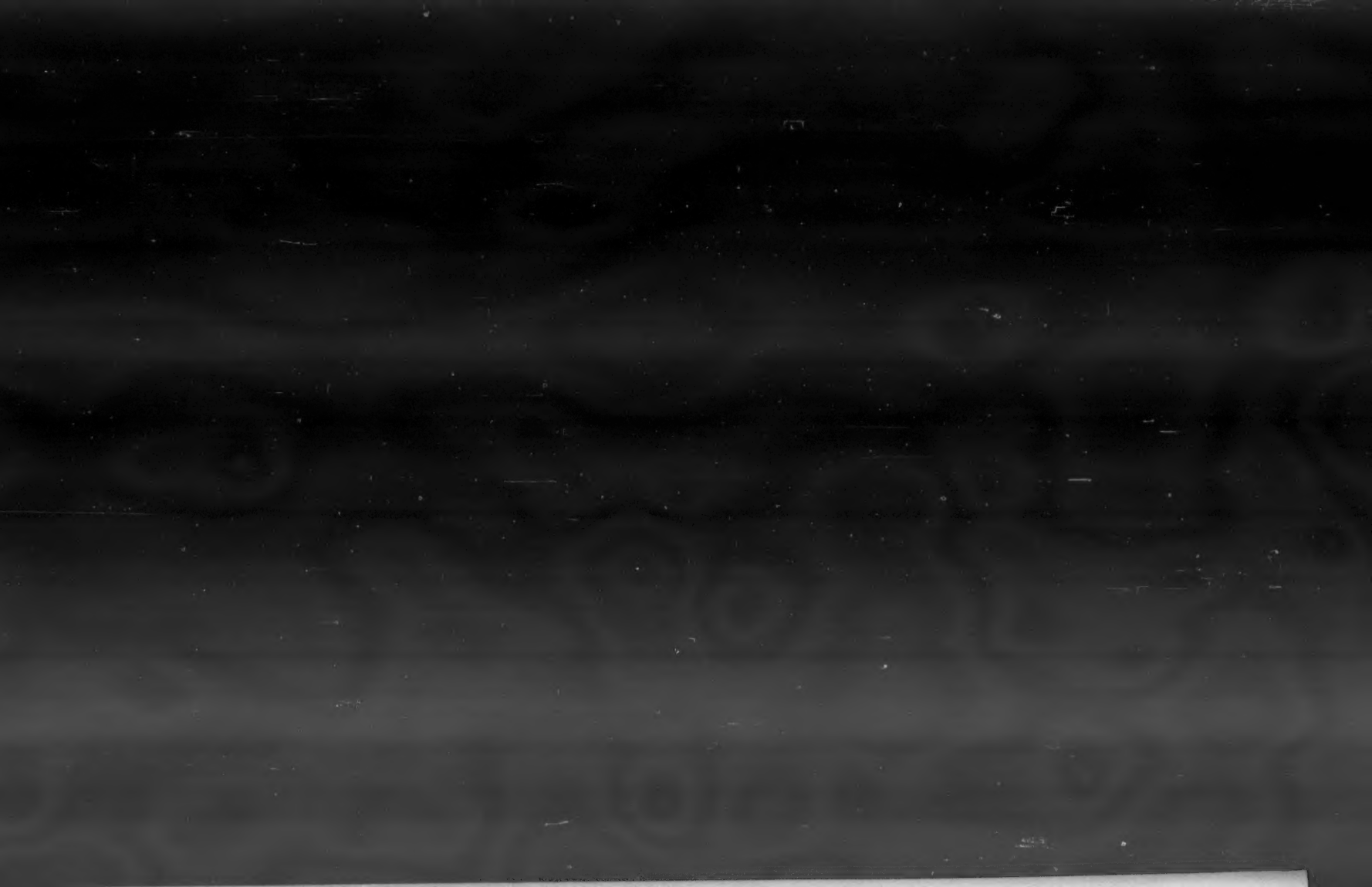
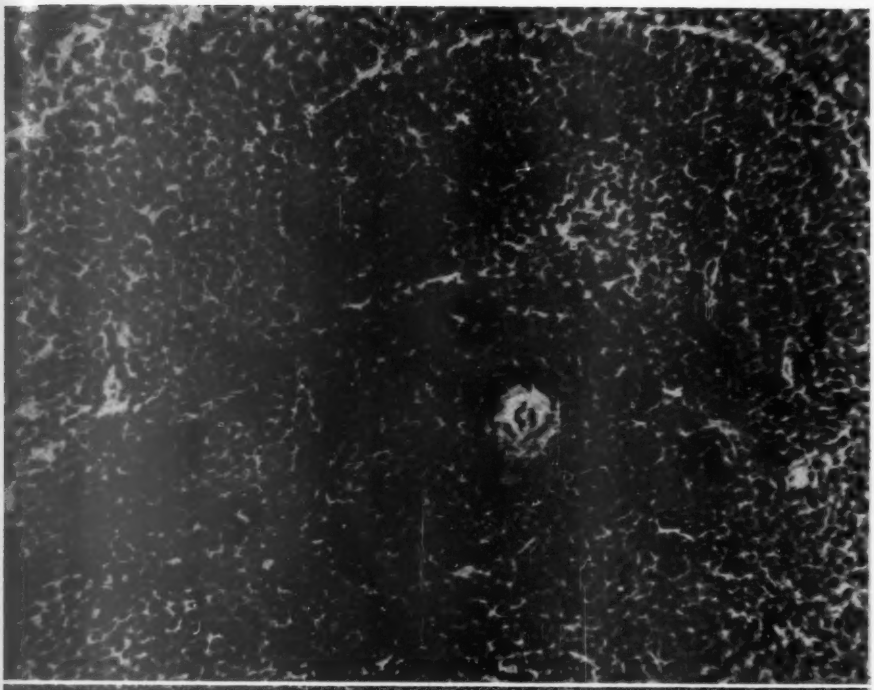


FIG. 9. Spleen from a rabbit in group F (also depicted in Fig. 5), 18 days after the administration of cortisone from days −5 to +12 and 10 mg. of BGG on day 0. A dark zone of lymphocytes, present in approximately normal quantities, surrounds the central arteriole. A pale, nonreactive area of reticuloendothelial cells is present at one side of the follicle. Antibody was not detectable in the serum. × 260.

FIG. 10. Spleen from a rabbit in group E, similar to that depicted in Figure 7. A greatly enlarged follicle is shown with an enlarged central area of modified reticular cells and intense mitotic activity. The central arteriole is located peripherally in a very scant intermediate zone of dark lymphocytes, surrounded by a narrow zone of monocytes. Antibody titers were maximal at this time. × 200.





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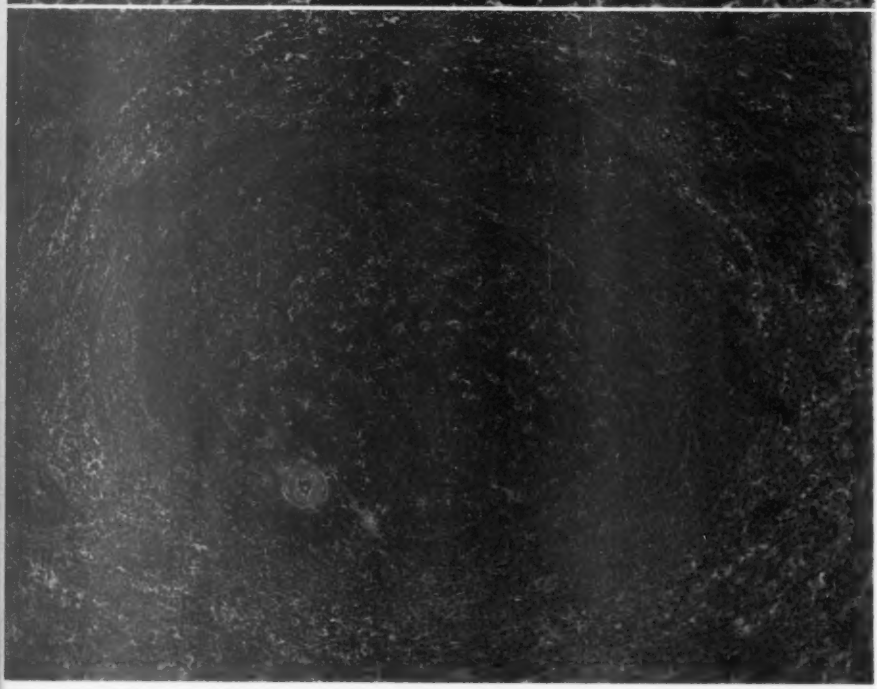

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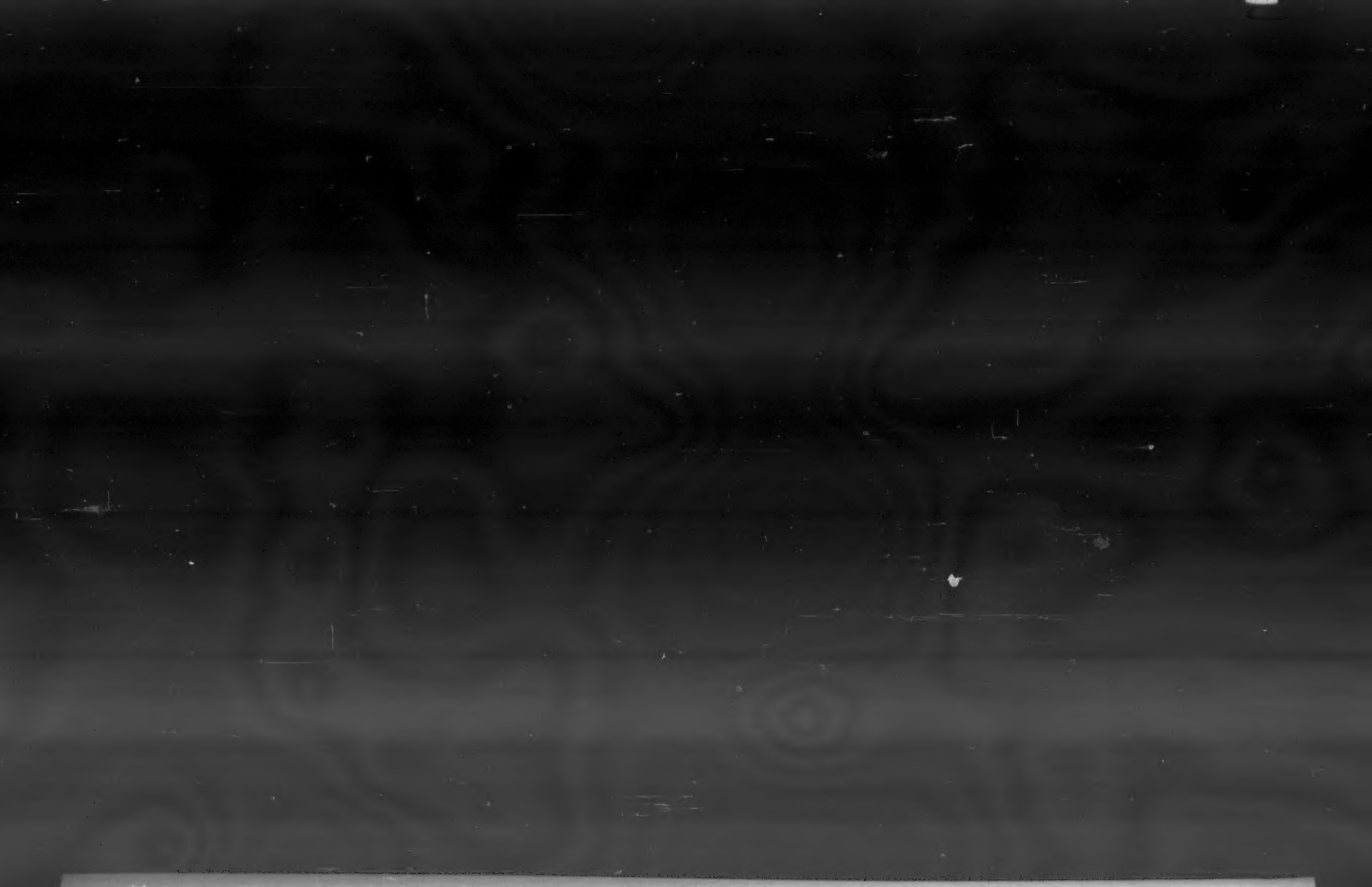
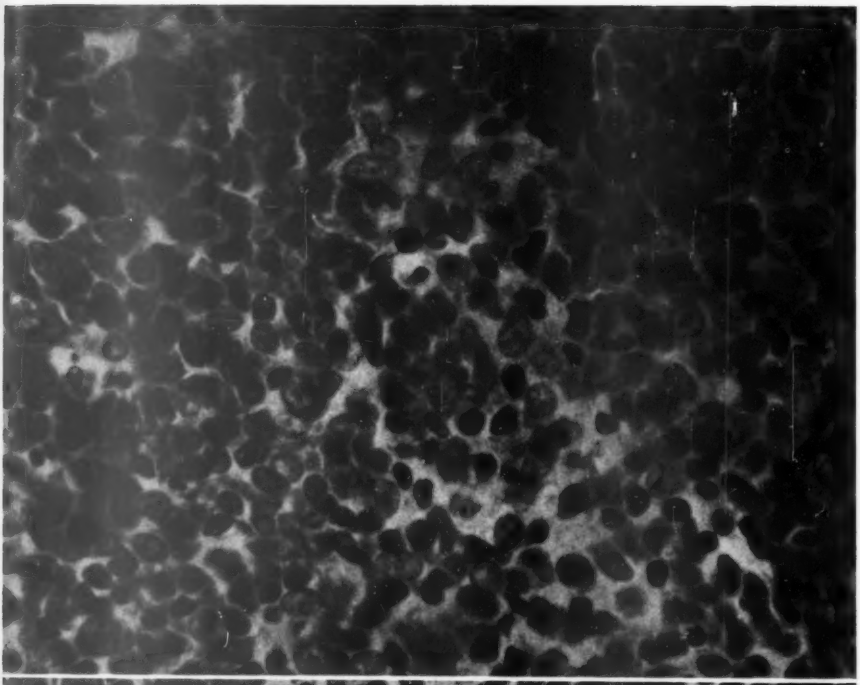
FIG. 11. Spleen from a rabbit in group E, similar to Figure 6. A small germinal center of a follicle is shown. Pale oval reticuloendothelial cells are evident with an occasional modified reticular cell, of which only a large oval nucleus with prominent nucleolus is identifiable. Dark, medium-sized, mature lymphocytes are noted peripherally, while lighter monocytic cells with a nuclear chromatin network are marginally arranged. × 620.

FIG. 12. Spleen from a rabbit in group E, higher magnification of the center of the active germinal follicle seen in Figures 7 and 10. The predominant element is a polygonal, modified reticular cell with a prominent nucleolus and chromatin pattern. Mitotic figures are prominent. × 620.





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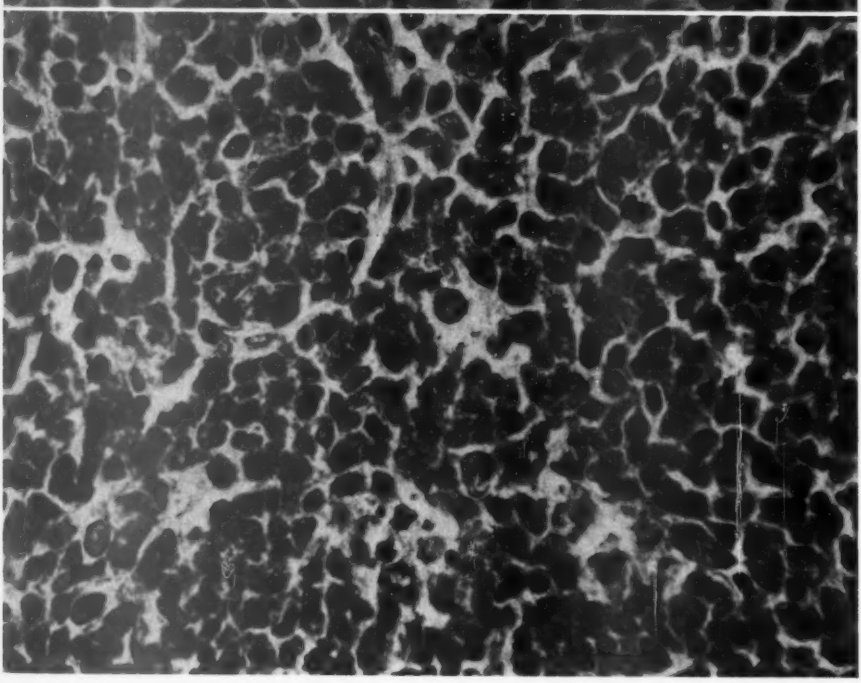

12

LESIONS OF TESTES OBSERVED IN CERTAIN PATIENTS WITH WIDESPREAD CHORIOCARCINOMA AND RELATED TUMORS

The Significance and Genesis of Hematoxylin-Staining Bodies in the Human Testis

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The significance of testicular scars as sites of healed primary gonadal tumors in patients with extragenital choriocarcinoma has long been known,[1] as has also the association of a small adult teratoma of testis with widespread metastasis.[2] A detailed study of a number of such cases from the files of the Testicular Tumor Registry of the Armed Forces Institute of Pathology yielded other interesting results which we believe merit publication.

Material

The 17 cases utilized in this investigation were those in which the patients had died with widespread tumors consisting of choriocarcinoma in 8 instances, embryonal carcinoma in 5, mixtures of choriocarcinoma and embryonal carcinoma in 3, and teratocarcinoma and embryonal carcinoma together in 1 case. The patients were all young men. Gynecomastia was reported in 11 patients, 7 of whom had choriocarcinoma. In all 17 the testicular lesion consisted only of a fibrous scar in which viable neoplastic tissue was either absent or of minute size (not exceeding 5 mm. in diameter). Testes containing a hemorrhagic focus in which little recognizable neoplasm was identifiable were excluded, since it is well known that these may represent instances of choriocarcinoma with scanty residual viable primary tumor tissue.[3] Also excluded were examples of minute teratoma of testis in patients who were not examined at necropsy.

As controls, the testes were examined from 60 patients with interstitial cell tumors, 20 patients with Sertoli cell tumors, 65 young patients dying from automobile and other accidents, 80 patients dying of malignant neoplastic disease of all types, and 25 patients with primary mediastinal teratoid tumors.

Pathologic Observations

The incidence and types of testicular lesions are shown in Table I. The most frequent lesion consisted of a more or less well-defined fibrous

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TABLE I

INCIDENCE AND TYPE OF TESTICULAR LESIONS

AFIP acc. no.	Shape of scar	Location	Hema-toxylin deposits	Sidero-phages	Teratomatous tissues	Atypical cells in surrounding tubules	Microscopic focus of seminoma	Histology of metastases	Gyneco-mastia
106546	Irregular	Not known	—	—	Epidermoid cyst, 3 mm. diam.	—	—	Embryonal carcinoma and chorio-carcinoma	+
132104	Irregular	Not known	+	+	—	+	+	Choriocarcinoma	+
142203	Rounded	Not known	+	++	Ruptured epidermoid cyst (with a few cholesterol clefts).	+	—	Embryonal carcinoma and chorio-carcinoma	+
150705	Irregular	Near rete	+	—	Three microscopic cysts, 2 lined by columnar epithelium, 1 without its lining.	+	+	Choriocarcinoma	+
161897	Irregularly linear	Near rete	+	+	—	—	—	Embryonal carcinoma and chorio-carcinoma	—
168079	Irregularly rounded	Upper pole, testis	—	++	—	+	—	Embryonal carcinoma	—
281811	Stellate	Near rete	—	+	—	+	—	Teratocarcinoma and embryonal carcinoma	—
293443	Stellate	Near rete	++	—	—	+	—	Embryonal carcinoma	—
323501	Irregularly rounded	On posterior surface of testis beneath tunica (relation to rete not determined).	++	—	—	+	—	Choriocarcinoma	—

335808	Elongated, bandlike	Near rete	+	−	−	+	−	Embryonal carcinoma	+
533124	Irregularly rounded	Near rete	++	−	−	−	−	Choriocarcinoma	+
589674	Irregular	Near rete	++	−	Microscopic squamous foci; mucous cyst; neuroglial tissue. Degenerating carcinomatous cells.	+	+	Choriocarcinoma	+
650216	Irregular	Not known	+	−	−	+	−	Embryonal carcinoma	−
658096	Irregularly rounded	Near rete	++	−	Eight or 9 microcysts (together measuring 3-4 mm. diam.). Lined by attenuated mucous cells or have desquamated lining. One mucous cyst surrounded by layer of smooth muscle.	+	−	Choriocarcinoma	+
747941	Elongated	Not known	−	+	Two epidermoid cysts, together measuring 3-4 mm. in length.	+	−	Choriocarcinoma	+
819008	Irregular	4 mm. from rete	++	−	One epidermoid cyst. A few malignant cells mixed with hematoxylin material.	+	+	Embryonal carcinoma	+
927471	Irregular	Near rete	+	+	Cluster of cysts, together measuring 3-4 mm. diam.; at least one lined by mucous cells with a few Kultschitzky cells.	−	−	Choriocarcinoma	+

scar (Fig. 1), often containing the ghostlike remains of hyalinized seminiferous tubules. The scar was usually irregular in shape, sometimes linear, wedge-shaped, or irregularly rounded. It consisted of relatively acellular collagenous tissue, usually dense but sometimes loose, and contained an occasional mast and plasma cell. In 10 of the 17 cases, the scar was situated in the neighborhood of the rete testis (Fig. 1), and in only 1 was it known to have been situated at the pole of the testis. Siderophages were present in 7 of the scars, in moderate numbers in 5 and in large numbers in 2 (Fig. 2).

In 13 of the cases, in addition to the scars, peculiar hematoxylin-staining deposits were observed (Figs. 1 and 3). These deposits appeared as rounded or ovoid foci sharply separated from one another by a basement membrane and intervening scar tissue. They were compactly arranged in a small portion of the scar or scattered uniformly through it. Their size and general configuration, and in particular the hyaline, thickened basement membrane surrounding each one, suggested strongly that the deposit was located within dilated seminiferous tubules (Fig. 3) and not in vascular channels. The hematoxylin-staining material was not accompanied by red cells, fibrin thrombi, or organizing thrombi; there was no smooth muscle in the surrounding wall. Hematoxylin-staining foci were present only in sites where seminiferous tubules were found and not in vascular channels. For further confirmation, one case (AFIP Acc. No. 589674) was examined in serial sections, and the hematoxylin deposits, Sertoli cells, and a corpus amylaceum were all observed within the same basement membrane. The deposits had an amorphous, granular, unorganized structure (Fig. 3). Sometimes they appeared as larger irregular fragments in an amorphous matrix.

In two instances, malignant neoplastic cells were recognizable within tubules, associated with the hematoxylin-staining substance (Fig. 4). These cells did not have the sharp outlines of the neoplastic cells in intratubular seminoma, and they had vesicular nuclear features suggesting undifferentiated tissue of the type seen in embryonal carcinoma.

The tissue around these aggregates was sometimes torn in the process of sectioning, suggesting calcification of the deposits. Von Kossa staining showed that phosphate was, in fact, often present, and staining with alizarin red established the presence of calcium. The hematoxylin staining was not, however, due to lime salts since calcium *per se* is not hematoxylinophilic.[4] The deposit often stained in positive manner and occasionally deeply with Mayer's mucicarmine. More significantly, Feulgen-positive[5] material was found frequently in these foci, indicating that mucoid substances and deoxyribonucleic acid (DNA) were responsible for most of the hematoxylin staining. Staining of paraffin

sections with Sudan black B [5] showed the presence of formol-fixed lipid in the deposits; these also gave a strongly positive reaction with Baker's acid hematein method.[5] The periodic acid-Schiff reaction was only weakly and variably positive. The Danielli coupled tetrazonium method [5] yielded a positive result, with a bright orange color in the tubular deposits, indicating the presence of tyrosine, tryptophan, or histidine. This occurred in spite of formalin fixation, which is likely to reduce the intensity of the reaction because of blocking in aromatic hydroxyl groups. Millon's reaction [5] demonstrated the presence of tyrosine. The hematoxylin deposits were composed, therefore, of an amorphous, complex mixture of phospholipid, protein, and scanty 1–2 glycol groups in combination with DNA. Some specimens, but not all, contained, in addition, mucicarminophilic material and calcium phosphate; calcium was not an essential part of the complex, however.

In addition to the scars and hematoxylin-staining bodies, certain neighboring viable tissues were seen. Teratomatous elements were present in 8 cases in close proximity to the scars. Although initially missed or interpreted as congenital rests, these consisted of epidermal cysts (Figs. 1 and 5) containing keratin in 4, mucous cysts in 2 (Fig. 6), and both types of cysts in another case; one contained 2 minute cysts lined by columnar non-mucus-secreting epithelium. In one of the lesions a mucous cyst was surrounded by a thick layer of smooth muscle. In the scar containing both mucous and epidermoid cysts, there was also some neuroglial tissue. A few typical enterochromaffin cells were present in the epithelial lining of one mucous cyst (Fig. 7).

In two cases (Acc. Nos. 168079 and 281811) the scars did not contain recognizable teratomatous tissue or hematoxylin-staining deposits. One was an irregularly rounded scar, 1.5 by 0.6 cm., near the upper pole of the testis, containing large numbers of siderophages (Fig. 2); the other was an irregularly stellate scar in the rete testis, containing few siderophages.

In 4 instances a microscopic focus of seminoma was encountered in the tissue surrounding the scarred area, in each case occupying no more than a fraction of a high-power field (Fig. 8). A far more prominent finding than these minute seminomatous foci was an alteration in the seminiferous tubules adjacent to or a short distance from the scarred area. These sometimes involved a considerable zone of tissue, and were evident in many blocks (Fig. 9). Where this change was present, spermatogenesis had ceased and the tubules usually showed hyalinization and contained only these atypical cells. Frequently there was an associated inner layer of Sertoli cells (Fig. 10) and at other times these filled the lumen.

The remaining portion of the testis usually showed atrophy of semini-

ferous tubules, hyalinization of basement membrane, and hyperplasia of interstitial cells.

Illustrative Cases

Two examples have been selected to illustrate the problems that may be encountered with this type of lesion.

Case 1 (AFIP Acc. No. 161897)

The patient was a 21-year-old white male. The first symptom was blood-stained sputum. Slight shortness of breath with chest pain developed within 1 month. Two months later he complained of pain in the right lumbar region. Shortness of breath became more marked, and he developed pain in the left lower chest. His appetite was poor, and he lost weight. Examination on admission revealed an acutely ill patient. The right apex posteriorly and the entire left lung field were dull to percussion. A large, hard, tender mass was palpable in the epigastrium. The clinical course was progressively downhill. A month later he became mentally confused and had severe coughing bouts, with expectoration of blood. He became comatose and died just over 4 months after his initial symptoms. Neither the correct diagnosis nor the possibility of a primary testicular neoplasm were suspected.

Necropsy revealed tumor deposits in the lungs, liver, ileum, and brain. The largest, weighing 220 gm. and measuring 9 by 6 by 5.5 cm., was situated in the retroperitoneal tissues. The mass lay to the right and below the junction of the second and third portions of the duodenum, and anterior to the right kidney and psoas muscle. It partially surrounded the inferior vena cava and aorta. The anterior wall of the vena cava was invaded by the neoplasm, which projected slightly into its lumen and was covered by a small amount of thrombus. On section, the mass had a variegated appearance, dark and light red with pale yellowish areas. The testes appeared normal in size and color and showed no macroscopic abnormality. Microscopic examination of the retroperitoneal mass and other tumor deposits showed embryonal carcinoma and choriocarcinoma. Sections of the testes were originally regarded as normal, and the possibility of a primary retroperitoneal teratocarcinoma with choriocarcinomatous differentiation was entertained. Re-examination of the testicular sections, however, showed a small linear scar containing intratubular hematoxylin-staining deposits. This was considered to be a healed primary testicular tumor.

Case 2 (AFIP Acc. No. 589674)

A 26-year-old white male had been in good health until 4 weeks before admission, when he complained of malaise, severe cramps, and some blood and mucus in the stools. He then noted left subcostal pain and persistent back pain. In the 4 weeks he lost 20 pounds. Severe anorexia and vomiting developed, and at the time of admission, severe pain over the sacrum and in the left flank were the main complaints. Inquiry elicited the information that about 4 or 5 years previously he had had some intermittent swelling and tenderness of both breasts.

Examination showed a thin man in moderate discomfort. There was tenderness over the epigastrium and left upper abdominal quadrant, and a nodular, hard mass, about 10 cm. in diameter, was palpable in the left hypochondrium. The testes appeared normal. There was bilateral gynecomastia, more pronounced on the right, with indurated breast tissue, 5 cm. in diameter, beneath the enlarged right nipple. Estimation of follicle-stimulating hormone excretion revealed 80 mouse units per 24 hours (normal for age, 8 to 20 units). Chorionic gonadotrophin level was 549,000 mouse units per 24 hours (normal, 0). Urinary estrogen assay was 34 gamma per 24 hours (normal for age, 2.5 to 10 gamma), and the 17-ketosteroid level was 17.1 mg. per 24 hours (normal, 10 to 20 mg.). Radiologic examination showed the stomach to be displaced to the right and anteriorly by a retroperitoneal mass in the region of the left kidney. Roentgenograms of the skull and lumbar spine revealed no lesions. A chest film showed many small, rounded deposits within the lung fields. Excretory urograms revealed a nonfunctioning left kidney.

A month after admission to the hospital the patient was treated with stilbestrol, 50 mg. 4 times daily. In an effort to determine the site of the primary lesion, 2 testicular biopsy examinations were made, but with negative results. The patient remained anemic, with a hemoglobin level below 8 gm. per 100 ml., despite blood transfusions. He became very dyspneic. Four hundred cc. of dark red blood was removed from the left pleural space. He remained in an oxygen tent until death, about 6 weeks after admission and 10 weeks after the onset of symptoms.

At necropsy, choriocarcinoma was found in the liver, lung, brain, kidney, spleen, vertebral bone marrow, and retroperitoneal soft tissue, including the left psoas muscle. There was bilateral gynecomastia. The right testis contained several reddish-yellow areas of discoloration and the left testis, many reddish foci. There was no tumor recognizable macroscopically, however, in spite of careful examination of many parallel slabs. Both testes contained zones of infarction, probably the result of surgical trauma. Leydig cell hyperplasia was more prominent in the left testis. In the region of the left rete testis a collagenous scar containing several minute squamous nests and cysts, one lined by mucous cells, was found along with some neuroglial tissue. There were hematoxylin-staining deposits and degenerating malignant neoplastic cells within a few tubules. The left testis is considered to be the primary site.

Discussion

Small focal scars secondary to trauma or orchitis are not unusual in the testes of adults. Circumscribed scars containing hemosiderin or calcification of the type reported by us, however, have not been observed except in patients with metastatic choriocarcinoma, embryonal carcinoma, or teratocarcinoma. Prym[1] is credited with the first report of a case of this type in which a testicular scar containing brown pigment was associated with choriocarcinomatous metastases. Heaney's case[6] was interpreted by him as an example of extragenital choriocarcinoma, but Symeonidis[7] and Roth[8] both regarded the hemorrhagic testicular scar as a retrogressed primary tumor. Michel[9] reported a further in-

stance, and Eck's first case[10] of testicular tumor with a pea-sized scar in the testis is probably similar. In Roth's series of testicular tumors,[8] a scar near the rete was associated with choriocarcinomatous metastases and gynecomastia in case 9. Roth mentioned intratubular calcification in his case. Friedman[11] referred to several scars of this type, with or without calcification. Rather, Gardiner and Frerichs[12] reported 6 new cases which, with the 18 they had accumulated from the literature, showed either a scar, foci of seminoma, or cysts and tubular structures in the zone of testicular fibrosis. Maluf, Loeffler and Erickson[13] also reported focal areas of fibrosis and patches of calcification associated with an adult testicular teratoma in a patient with widespread choriocarcinoma. More recently Maring, Knopp and Langecker[14] also considered a scar in the testis as primary in a patient who died of widespread choriocarcinoma.

Calcification is not unusual in the testis. The most common forms of calcified bodies are mucospherules or concretions found in atrophic seminiferous tubules (Fig. 11). These are smoothly rounded bodies with a concentrically laminated structure; they occupy the tubular lumens and are occasionally surrounded by a single residual layer of Sertoli-like cells. These psammoma-like bodies are seen frequently in cryptorchid testes and, in these, they are associated with the multiple tubular "adenomas" or foci of Sertoli cell hyperplasia common in undescended testes (Fig. 12). The concretions are also observed in atrophic seminiferous tubules surrounding any type of testicular neoplasm, and in these circumstances may become "caught up" in the neoplastic process. These bodies are probably related to abnormal activity of Sertoli cells. They are somewhat analogous to corpora amylacea in the prostate or lung. The mucospherules may be either eosinophilic or hematoxylinophilic. They stain feebly with mucicarmine; sometimes a small central core appears carminophilic while the periphery remains unstained. On the other hand, they give an intensely positive PAS reaction. Even when the calcified bodies are fragmented in the process of sectioning, their laminated structure is usually still discernible.

Less frequently, calcific deposits may be seen in healed inflammatory lesions and in certain primary tumors of the testis in the form of granular calcium salts.

In contrast to these, the amorphous and granular hematoxylin-staining bodies described in this investigation and not seen in any of the control testes are quite distinct from the corpora amylacea and the calcifications of inflammatory origin. By histochemical technique we have demonstrated that these hematoxylin bodies are composed of a complex mixture of phospholipid, protein and scanty 1-2 glycol groups, DNA and

calcium phosphate. Calcium, while present, is not an essential component. It may be safely assumed, therefore, that the hematoxylin bodies represent necrosis of nuclear chromatin. That such a possibility does exist is suggested by Friedman's observation [11] that choriocarcinoma, by virtue of its tendency to blood vessel invasion, may cause infarction of considerable extent. The intratubular neoplastic tissue accompanying the hematoxylin-staining material, seen in 2 of our cases, represents an undifferentiated form of malignant tumor. It may well be that the high metabolic turnover of such neoplastic cells, perhaps, associated with a poor blood supply, leads to massive necrosis of the intratubular tumor.

The significance of the hematoxylin-staining bodies was further investigated. They were not infrequently observed in grossly recognizable testicular teratoid tumors (Figs. 13 and 14) not included in this study of 17 cases. In a number of these the transformation of recognizable intratubular malignant elements to granular debris could be traced. It is significant that in these cases the neoplastic cells were undifferentiated and did not resemble either germ or seminoma cells.

In 8 of our patients minute teratomatous structures were observed. These were similar to those reported by Stärk,[2] Craver and Stewart,[15] Symeonidis,[7,16] Rottino and DeBellis,[17] and Riopelle.[18] In 4 patients microscopic seminomas were associated with hematoxylin-staining bodies and teratomatous structures.

Another frequent finding was a peculiar alteration observed in 13 cases. This was characterized by large hyperchromatic cells with a small amount of vacuolated cytoplasm. These cells occurred inside seminiferous tubules and resembled germ cells. They were identical to the cells described by Dixon and Moore [8] in the rete testis of some of their examples of invasive seminoma. Similar cells were not observed in any of our control specimens; we have seen them most frequently in the seminiferous tubules of patients with seminoma and occasionally they have been associated with other germ cell tumors of the testis. Their significance and possible relationship to the genesis of teratoid tumors of testes is under investigation.

Thus, in a group of 17 patients, 11 with gynecomastia, widespread metastases of either choriocarcinoma or embryonal carcinoma have been observed at necropsy. In these cases certain lesions have been observed in the testis, consisting of a circumscribed scar and intratubular hematoxylin-staining material with a high DNA content. These have sometimes been accompanied by small teratomatous structures and in a few cases by microscopic foci of seminoma. The occurrence of these testicular structures in patients with generalized metastatic growths would

appear to provide satisfactory circumstantial support for the theory of retrogression in primary testicular tumors.

The appearance of hematoxylin-staining bodies in well defined teratoid testicular tumors leaves no doubt that they represent cellular debris resulting from ischemic necrosis of neoplastic tissue. These deposits are located almost exclusively within tubular structures. Although dilated, the tubules are most probably either seminiferous tubules or ducts. It is possible that necrotic debris in this location is removed with difficulty and forms an inspissated matrix in which calcium salts may be deposited.

Although the 17 cases investigated were all examined at necropsy, and the testicular lesions observed were not found in 250 control testes, it cannot be assumed that such lesions always lead to generalized dissemination of tumor. It must be borne in mind that these lesions were frequently detected only after careful search and special study of the testes in patients who had metastatic tumors of apparent germ cell origin. It is possible that a number of these testicular lesions, in fact, may remain quiescent and never give rise to metastases. Similarly, it may be that in patients without gynecomastia or metastatic choriocarcinoma but with generalized metastases of less distinctive germ cell tumors of other types, the testes are either superficially examined or not examined at all. Thus, a minute primary tumor, whether viable or having undergone retrogression, may be missed. The high coincidence of choriocarcinoma and gynecomastia in this group of cases may, therefore, be genuine or reflect case selection.

Our observations have a bearing on the question of the existence of primary extragonadal choriocarcinoma in the male. Cases have been reported in the retroperitoneum and the peritoneal cavity, in the mediastinum, and in the pineal, as well as in various organs.[19–22] We believe that skepticism is justified in most cases of this type. This applies particularly to the cases [6,23,24] in which incidental lesions found in the testes should probably be re-interpreted as examples of neoplastic retrogression. We consider it essential that the testes from patients of this type be carefully and serially examined. Only if no lesions are found may the case be accepted as an example of extragonadal choriocarcinoma.

So far there have only been about 30 cases reported in which careful examination of the testes showed no probability of primary neoplasm in this location.[19,20,22,25–27] Stowell, Sachs, and Russell,[22] however, have questioned (a) the evidence indicating spontaneous healing of primary chorionepithelioma while the metastases continue to grow, and (b) the significance of scars in the testes. Lynch and Blewett[21] have offered the intriguing possibility that "testicular scars" (reported as the alleged site of burned-out primary tumors in patients with widespread chorio-

carcinomas) "might themselves be the results of extragenital growths" and that "the intense hormonal stimulation may cause totipotent gonadal cells to develop into small teratomata." While absolute proof may be lacking, we believe that the testicular scars and the other lesions of the type described by us constitute the primary foci from which widespread dissemination of choriocarcinoma and related tumors may occur. Similar lesions were not observed in any other condition or in the contralateral unaffected testes of patients with metastatic choriocarcinoma or in other patients with primary extragonadal choriocarcinoma.

The intratubular location of the hematoxylin-staining bodies as well as the appearance of malignant neoplastic cells in the tubules offers evidence in support of an intratubular origin of teratoid testicular tumors, a concept already accepted for seminoma.[3,28] A secondary extension into the tubules in 13 of 17 cases by such minute foci of neoplastic tissue would seem highly unlikely. In seminoma it is often possible to trace the tubular origin of the lesion even when the tumor has attained a considerable size. In embryonal carcinoma and other teratoid testicular tumors, this feature is rather rare unless the tumor is seen very early or is undergoing regression. The long-standing controversy over the origin of testicular teratoid tumors mainly centers about the concept of escape by embryonic median or paramedian tissue from the influence of a primary organizer [29] versus the germ cell theory of origin. The presence of genuine choriocarcinoma in teratoid testicular tumors favors the latter hypothesis, and the possibility of origin of these tumors by fusion of haploid cells has received great support from recent work on the nuclear sexing of the neoplastic tissue.[30] The tubular localization of testicular teratoid neoplasms which have undergone partial regression suggests a probable tubular origin for embryonal carcinoma and related tumors, an observation which further supports the concept of germ cell origin for these neoplasms.

Summary

In 17 patients with widespread choriocarcinoma and related neoplasms, frequently with associated gynecomastia, certain lesions have been observed in the testes. In all instances a distinct and well defined fibrous scar was found. In addition, in 13, peculiar amorphous hematoxylin-staining deposits were observed in dilated seminiferous tubules. By histochemical methods, these deposits were shown to consist of phospholipid, protein debris, and DNA, and in some cases, mucoid substances and calcium phosphate. Believed to originate from the necrosis of undifferentiated neoplastic tissue of germ cell origin, the presence of these hematoxylin deposits lends support to earlier reports of burned-out

primary testicular tumors. In 8 cases there were remnants of mature teratoma, and in 4 there were microscopic foci of seminoma in relation to the scars.

The observations are interpreted to indicate regression of primary testicular tumors. Biologic factors responsible for regression in a primary neoplasm remain to be established. Certain alterations were also observed in the germ cells of the testis adjacent to the scar; the significance of these could not be determined.

References

1. Prym, P. Spontanheilung eines bösartigen, wahrscheinlich chorionepitheliomatösen Gewächses im Hoden. *Virchows Arch. path. Anat.*, 1927, 265, 239–258.
2. Stärk, A. Malignes Chorionepitheliom bei einem 28jährigen Soldaten mit kleinem Embryom des Hodens. *Frankfurt. Ztschr. Path.*, 1918, 21, 142–162.
3. Dixon, F. J., and Moore, R. A. Tumors of the Male Sex Organs. Section VIII, Fascicle 32, Atlas of Tumor Pathology. Subcommittee on Oncology of the Committee on Pathology of the National Research Council, Armed Forces Institute of Pathology, Washington, D.C., 1952, p. 59.
4. Azzopardi, J. G. Oat-cell carcinoma of the bronchus. *J. Path. & Bact.*, 1959, 78, 513–519.
5. Pearse, A. G. E. Histochemistry, Theoretical and Applied. Little, Brown & Co., Boston, 1953, p. 415.
6. Heaney, H. G. Extragenital chorionepithelioma in the male. *Am. J. Cancer*, 1933, 19, 22–30.
7. Symeonidis, A. Über das Chorionepitheliom beim Mann und seine hormonale Wirkung in Form von "Schwangerschaftsveränderungen." *Beitr. path. Anat.*, 1934, 94, 370–375.
8. Roth, F. Über die bösartigen Hodengewächse, insbesondere das Chorionepitheliom und die Möglichkeit der Spontanheilung des primären Hodenteratoids, mit einem Beitrag zur Frage des Diabetes insipidus. *Ztschr. Krebsforsch.*, 1950, 57, 21–69.
9. Michel, G. Zur Frage der sogenannten extragenitalen Chorionepitheliome. *Frankfurt. Ztschr. Path.*, 1947, 59, 59–68.
10. Eck, H. Über Spontanheilung bösartiger Geschwülste, besonders des malignen Hodenteratoms. *Zentralbl. Chir.*, 1952, 77, 2240–2248.
11. Friedman, N. B. The comparative morphogenesis of extragenital and gonadal teratoid tumors. *Cancer*, 1951, 4, 265–276.
12. Rather, L. J.; Gardiner, W. R., and Frerichs, J. B. Regression and maturation of primary testicular tumors with progressive growth of metastases; report of 6 new cases and review of literature. *Stanford M. Bull.*, 1954, 12, 12–25.
13. Maluf, N. S. R.; Loeffler, R. K., and Erickson, E. E. Effect of bilateral adrenalectomy on metastatic choriocarcinoma from teratoma of the testis. *J. Clin. Endocrinol.*, 1956, 16, 1217–1226.
14. Maring, H.; Knopp, J., and Langecker, H. Chorionepitheliom beim Manne. *Acta endocrinol.*, 1956, 21, 289–298.
15. Craver, L. F., and Stewart, F. W. An unusual case of teratoma testis. *J.A.M.A.*, 1936, 106, 1802–1804.

16. SYMEONIDIS, A. Betrachtungen über das Hodenteratoid, seine Metastasierung und das sog. "extragenitale" Chorionepitheliom an Hand eines narbigen stecknadelkopfgrossen rudimentären Hodenteratoids mit dreikeimblätterigen und Chorionepitheliommetastasen. *Virchows Arch. path. Anat.*, 1943, **311**, 509–518.

17. ROTTINO, A., and DEBELLIS, H. Extragenital chorioma: its relation to teratoid vestiges in the testicles *Arch. Path.*, 1944, **37**, 78–80.

18. RIOPELLE, J. L. Le chorio-épithéliome extragénital et ses relations avec un tératome latent du testicule. *Union Med. Canada*, 1948, **77**, 1399–1407.

19. MAGOVERN, G. J., and BLADES, B. Primary extragenital chorioepithelioma in the male mediastinum. *J. Thoracic Surg.*, 1958, **35**, 378–383.

20. GUICHARD, A., and CABANNE, F. Contribution à l'étude des chorio-épithéliomes pulmonaires chez l'homme et de leurs formes dites primitives. *J. méd. Lyon*, 1953, **34**, 235–265.

21. LYNCH, M. J. G. and BLEWETT, G. L. Choriocarcinoma arising in the male mediastinum. *Thorax*, 1953, **8**, 157–161.

22. STOWELL, R. E.; SACHS, E., and RUSSELL, W. O. Primary intracranial chorionepithelioma with metastases to the lungs. *Am. J. Path.*, 1945, **21**, 787–801.

23. BONN, H. K., and EVANS, N. Extragenital chorioepithelioma in the male with associated gynecomastia; report of a case. *Am. J. Surg.*, 1942, **58**, 125–132.

24. CHERNOFF, H. M.; EVANS, T. S.; BARTLETT, C. J., and SWIRSKY, M. Y. Extragenital chorionepithelioma in the male. *Arch. Int. Med.*, 1945, **76**, 347–351.

25. KRIVAK-KNEZEVIĆ, Š. Ein Fall von extragenitalem Chorionepitheliom bei einem Manne. *Med. Klin. Berl.*, 1958, **53**, 1369–1371.

26. PORTMAN, J. Über das ektopische Chorionepitheliom beim Mann. *Beitr. path. Anat.*, 1959, **120**, 474–482.

27. SCHÄFER, H. Extragenitales Chorionepitheliom bei einem Mann. *Strahlentherapie*, 1959, **108**, 283–287.

28. WILLIS, R. A. Pathology of Tumours. C. V. Mosby Co., St. Louis; Butterworth & Co., London, 1953, ed. 2, p. 560.

29. WILLIS, R. A. The Borderland of Embryology and Pathology. Butterworth & Co., London, 1958, p. 450.

30. THEISS, E. A.; ASHLEY, D. J. B., and MOSTOFI, F. K. Nuclear sex of testicular tumors and some related ovarian and extragonadal neoplasms. *Cancer*, 1960, **13**, 323–327.

[*Illustrations follow*]

Legends for Figures

All photographs were prepared from sections stained with hematoxylin and eosin.

Fig. 1. (AFIP Acc. 589674, Neg. 59–3809) A hyalinized scar adjacent to the rete testis contains hematoxylin-staining bodies and a cluster of small cysts. × 20.

Fig. 2. (AFIP Acc. 168079, Neg. 60–113) A scar contains numerous siderophages. Note the fibrosed remnant of seminiferous tubules. × 130.

Fig. 3. (AFIP Acc. 323501, Neg. 59–6417) Amorphous granular, nonlaminated hematoxylin-staining material appears in the tubules. Note the hyalinized basement membrane and the complete absence of red or white cells. × 350.

Fig. 4. (AFIP Acc. 589674, Neg. 59–3806) Tubules contain both fragmented hematoxylin bodies and neoplastic cells. The cells resemble an undifferentiated embryonic type of carcinoma. × 165.

Fig. 5. (AFIP Acc. 589674, Neg. 59–6415) Higher magnification of area "A" in Figure 1, showing a cyst lined by squamous cells. × 440.

Fig. 6. (AFIP Acc. 589674, Neg. 59–6414) Higher magnification of area "B" in Figure 1, showing a cyst lined by tall columnar mucous epithelium. × 440.

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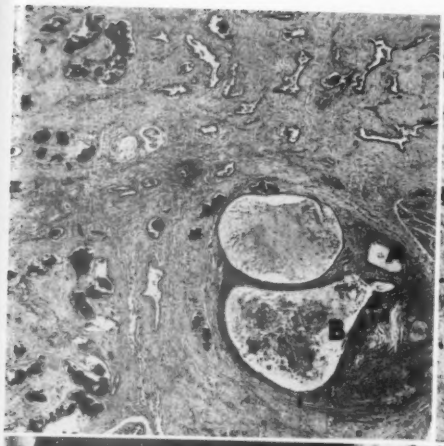

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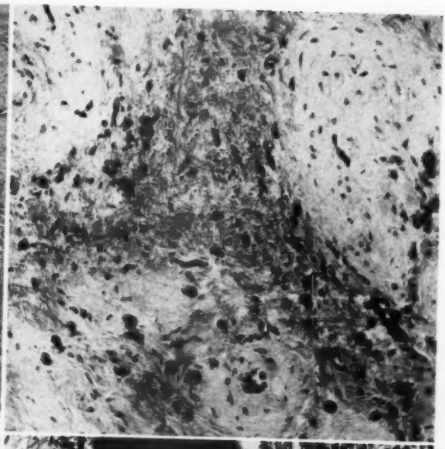

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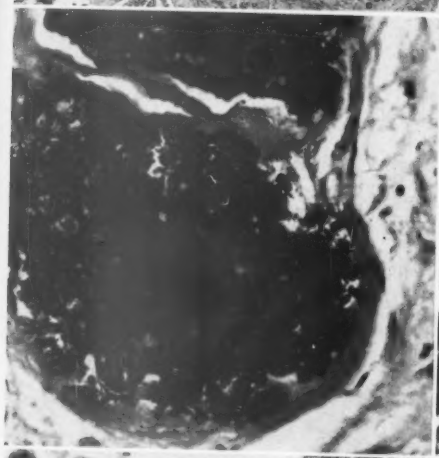

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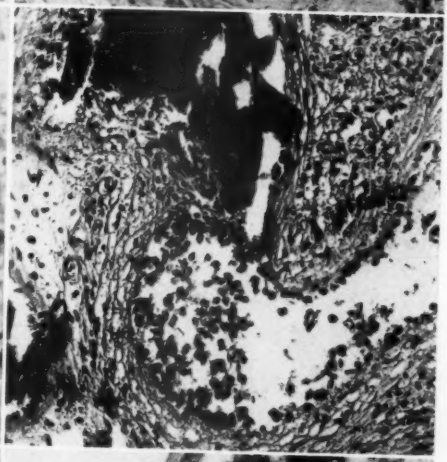

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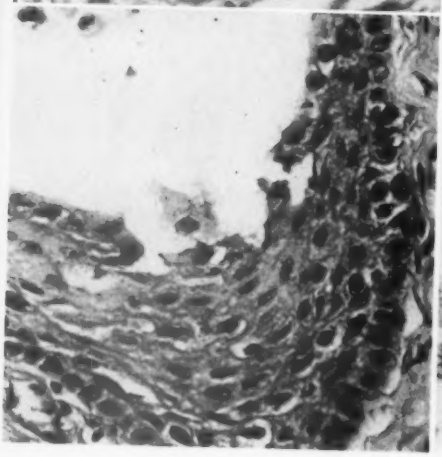

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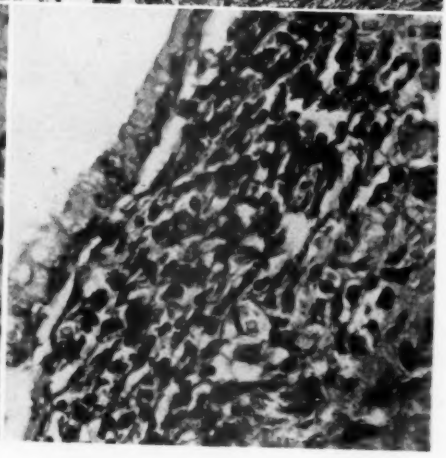

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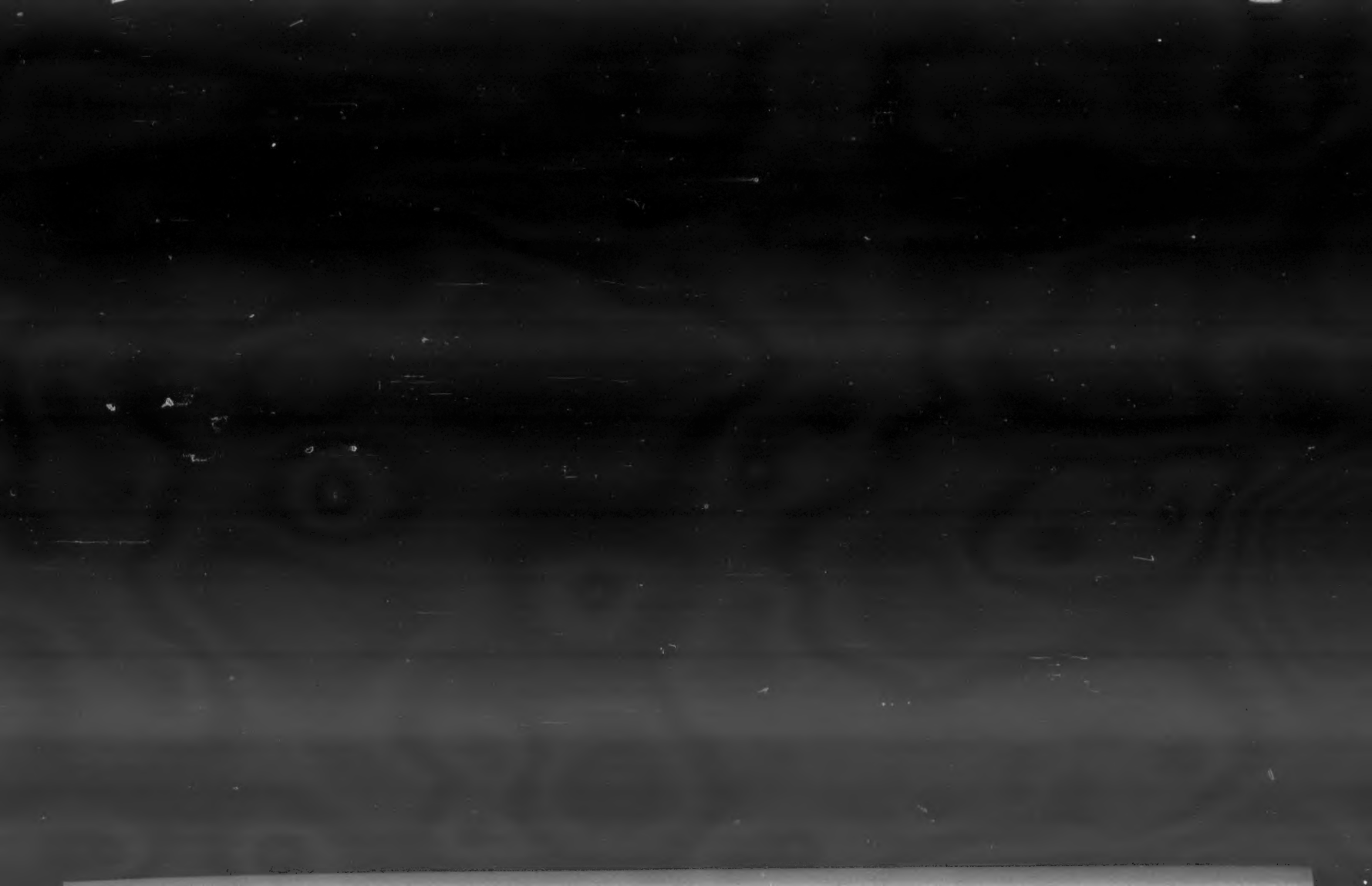
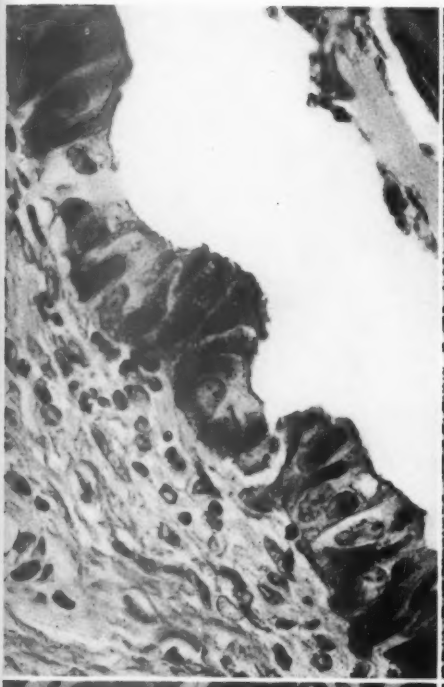
FIG. 7. (AFIP Acc. 927471, Neg. 59–5982) A mucous cyst from another case, showing enterochromaffin cells wedged between the bases of the columnar cells. × 530.

FIG. 8. (AFIP Acc. 589674, Neg. 59–5977) A small focus of typical seminoma from an area adjacent to a testicular scar. The cells are vacuolated and appear mostly in small groups. × 165.

FIG. 9. (AFIP Acc. 323501, Neg. 59–6419) Altered, hyperchromatic germ cells in the tubules surrounding a scar. × 130.

FIG. 10. (AFIP Acc. 323501, Neg. 59–6418) Higher magnification of Figure 9, showing hyperchromatic cells and vacuolation of the cytoplasm. Note the arrangement of Sertoli cells between the hyperchromatic abnormal germ cells and the lumen of the tubules. × 660.





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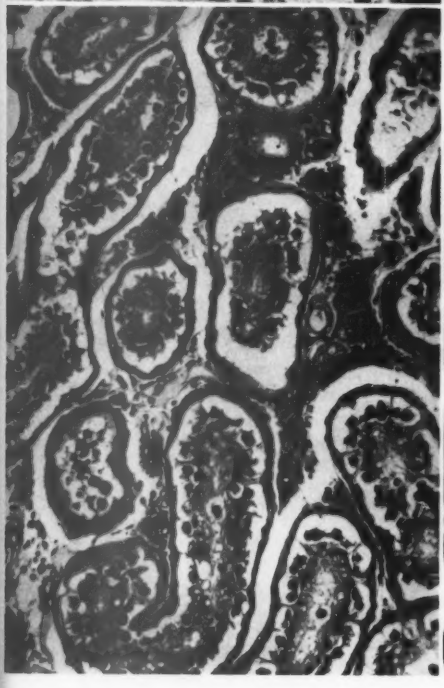

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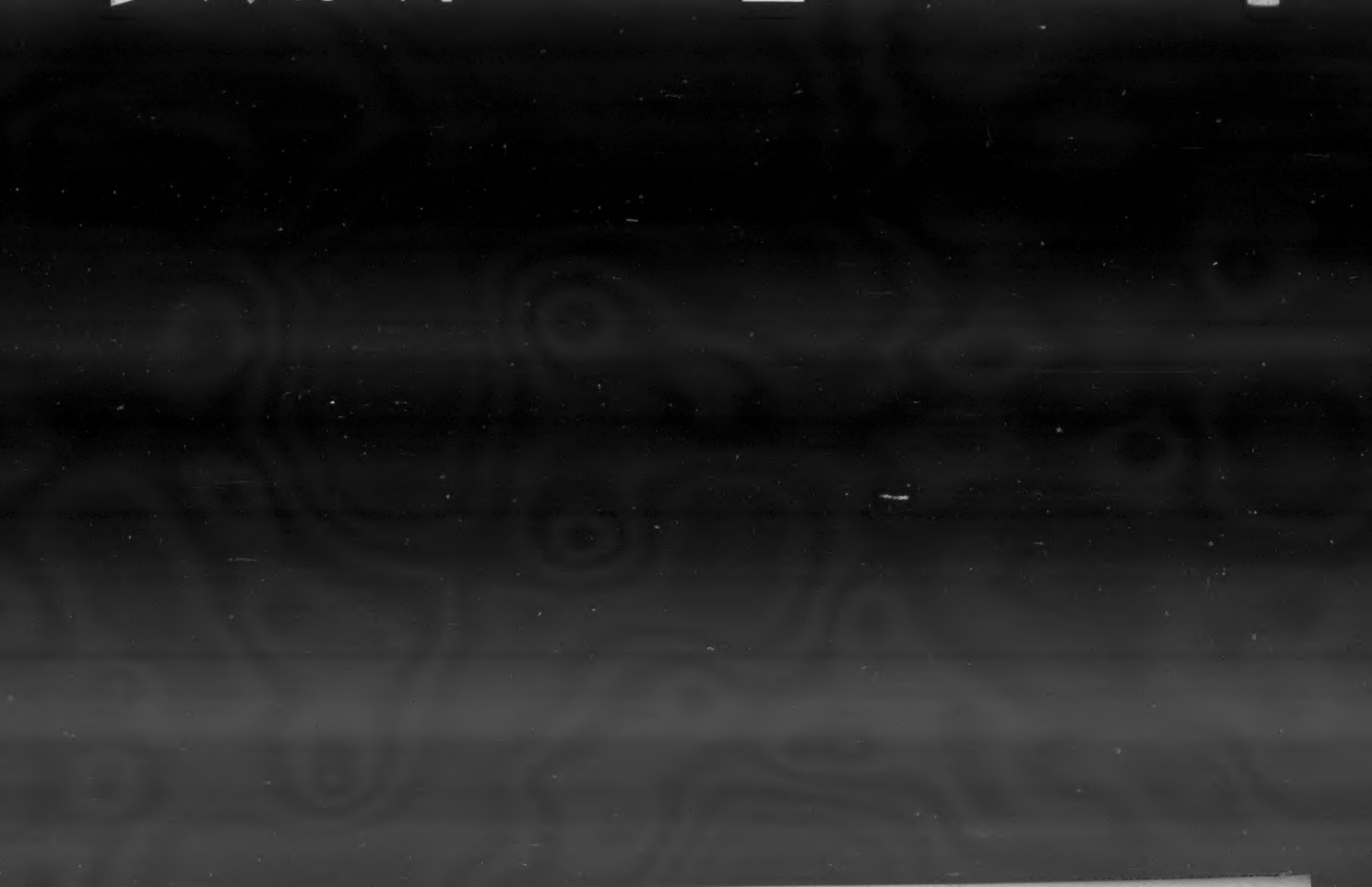
FIG. 11. (AFIP Acc. 323501, Neg. 59–5623) The structure of the mucospherules frequently observed in testes is shown. Note the concentric laminated appearance. × 305.

FIG. 12. (AFIP Acc. 307805, Neg. 59–3985) A solitary mucospherule frequently seen among persistent Sertoli cells in an undescended testis. × 195.

FIG. 13. (AFIP Acc. 480273, Neg. 59–4402) A teratoma of the testis, consisting chiefly of ossifying cartilage and adipose marrow. Note the hematoxylin-staining deposits in the adjacent scarred area. × 50.

FIG. 14. (AFIP Acc. 532644, Neg. 59–6422) A control case with grossly detectable embryonal carcinoma. In one area a typical hematoxylin-staining deposit is observed in intimate relationship to the neoplasm. This is believed to represent an area of necrosis in the tumor. × 42.



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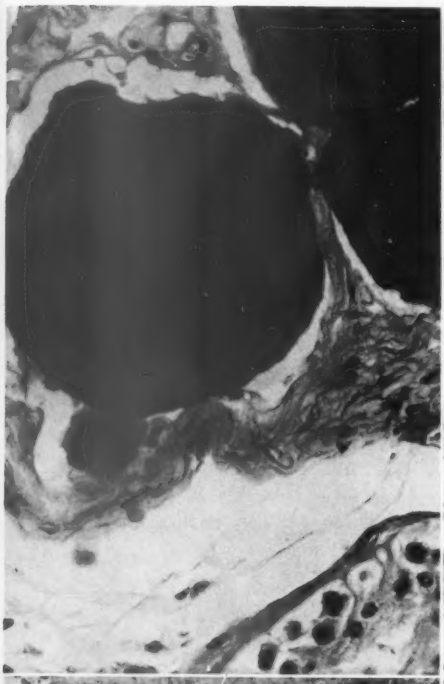

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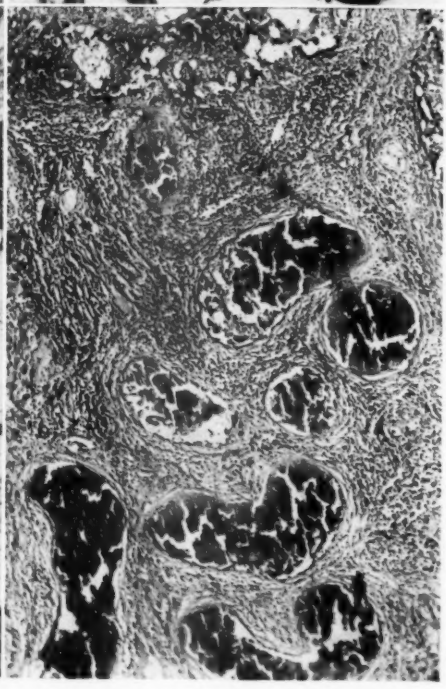

14

JUXTAGLOMERULAR CELL COUNTS AND HUMAN HYPERTENSION

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The juxtaglomerular body of the kidney was classically described by Goormaghtigh.[1] It consists of 8 to 15 epithelioid cells located near the glomerular hilus, apparently in continuity with the muscle cells of the afferent arteriole, and in close proximity to the macula densa. Together, the juxtaglomerular body and macula densa compose the juxtaglomerular apparatus.[2] The cells described in this paper do not include those of the macula densa. The function of these morphologic entities is not known with certainty, although the juxtaglomerular cells have been thought by many to have an endocrine action. In recent years, attempts have been made by Hartroft and associates,[3-5] Tobian,[6] and others[7-12] to correlate the granularity of the renal juxtaglomerular cells with hypertension, blood electrolytes, and the effects of mineralocorticoid hormones. Most of the work has been done with experimental animals. An attempt was therefore made to correlate the morphologic features in human surgical specimens with clinical observations in patients with hypertension. Cases of hypertension following partial or complete occlusion of a renal artery, so-called "Goldblatt kidneys," were investigated first because most physicians accept these as clear-cut examples of human hypertension primarily of renal origin.

MATERIAL AND METHODS

Surgically removed human kidney tissue was investigated in 112 cases. This included: (a) 10 "control" specimens from normotensive patients. The kidneys were removed because of renal hemorrhage, cysts, hydronephrosis, calculi or carcinoma; (b) 20 "Goldblatt type" kidneys without pyelonephritis. In 5 of these a biopsy of the contralateral kidney was made at the time of operation; (c) 18 kidneys with chronic pyelonephritis, including 8 from patients with persistent hypertension (blood pressure levels of 150/90 or more) and 10 from patients without hypertension; (d) 37 biopsy specimens from patients who had sympathectomy operations for "essential" hypertension. The degree of arteriolosclerosis in these specimens was designated grade 0, I, II and III, according to criteria described previously[13]; (e) 10 renal biopsy specimens from patients in whom a diagnosis of "malignant" hypertension was based on clinical data or histologic evidence of arteriolar necrosis, or both; (f) 7 renal specimens from patients who had removal of a pheochromocy-

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toma; and (g) 10 kidney biopsy fragments showing subacute or chronic glomerulonephritis.

All the renal biopsy tissues were procured from adult patients, and were obtained during an "open" surgical exposure; they averaged 1.2 by 0.8 by 0.4 cm. The operative specimens were submitted, for the most part, by Dr. Reginald H. Smithwick, Dr. William Porell and their associates at the Massachusetts Memorial Hospitals, Boston. Additional tissue was kindly provided by Dr. Gustave J. Dammin, Peter Bent Brigham Hospital, Boston; Dr. Louis B. Thomas, Bethesda Clinical Center, Bethesda, Maryland; Dr. S. Bernstein, Veterans Administration Hospital, Philadelphia; and Dr. Gordon F. Vawter, Children's Medical Center, Boston. Several specimens were discarded because of inadequate size, preservation or fixation, or incomplete clinical investigation. Florence W. Haynes, Ph.D., had performed renin assays on renal venous blood obtained at operation in 13 of the 20 "Goldblatt type" cases.

Tissues were usually fixed in Zenker's fluid, but the juxtaglomerular cell granules appeared better defined in a few instances when formalin followed by Bouin's fixative was used. Microscopic diagnoses in each case were made from conventional sections stained with hematoxylin and eosin. Special stains were then applied. The periodic acid-Schiff (PAS) stain, Masson's trichrome with ponceau fuchsin, originally used by Goormaghtigh, and such methods as the Gram or Wilson stains,[14] using ethyl violet, were employed. The Bowie stain utilized by Pitcock and Hartroft,[5] and composed of a mixture of Biebrich scarlet and ethyl violet, was found to be most useful in delineating the architecture, and particularly the granules of the juxtaglomerular cells. With this stain (Fig. 1), deep purple granules can be identified in the juxtaglomerular cells and in certain cells of the convoluted tubules also (Fig. 2). The elastica of the arteries stains purple, while other tissues are pink.

Estimates of the activity in juxtaglomerular cells were made in the above cases, first using the juxtaglomerular index (JGI) devised by the Hartrofts.[3,4] In general, this was increased in the "Goldblatt type" kidneys. Since, however, it is mostly an estimate of intracellular granularity, it did not reflect the presence of increased numbers of cells or of large, clear, nongranulated, apparently hypertrophied cells often found in abnormal kidneys. To express adequately these fairly frequent variations in the size and number of cells, a new counting method, the juxtaglomerular cell count (JGCC) was devised.

For the latter, the total number of cells in 25 juxtaglomerular bodies was counted, and their types were tabulated. Four cell types were commonly identified: type I, small cells with little or no granulation; type II, small cells with moderately granulated cytoplasm; type III, cells with heavily granulated cytoplasm, usually somewhat larger than type II; and type IV, large agranular cells with clear, watery cytoplasm.

The granularity of convoluted tubular epithelium was also assessed by counting 1,000 cells, using every fifth field and every fifth row under high dry magnification. Granularity was graded as follows: 0, no granules; +, slight granularity; ++, moderate to heavy granularity. The tubular epithelium was often found to contain more granularity in abnormal kidneys than in the controls. However, the differences were not statistically significant and the results are not included in this paper. Only the results with a probability value (p) smaller than 0.05 will be discussed, unless otherwise specified.

The cases were investigated histologically by two independent observers, and paired data analyses did not disclose any significant difference in their results. Clinical correlations were made only after completion of the histologic study.

Results

In control kidneys from normotensive patients, cell types I and IV together accounted for 95 per cent of the total juxtaglomerular cells. The

TABLE I

JUXTAGLOMERULAR CELL TYPES AND TOTAL COUNTS IN SURGICALLY REMOVED HUMAN KIDNEYS

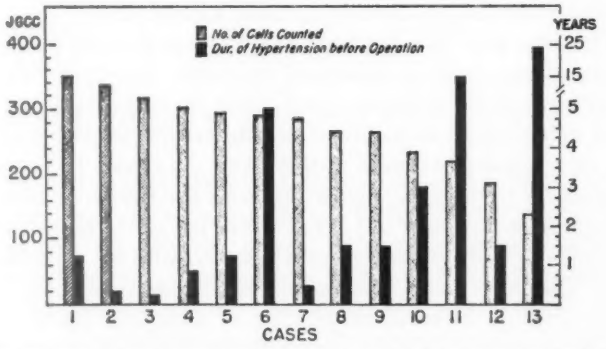
	No. of cases	JGCC * (total)	Types of cells II and III	Types of cells IV
Controls	10	192 ± 21	9 ± 7	27 ± 21
"Goldblatt": Total	20	273 ± 53	29 ± 18	20 ± 13
Cured (11)		294 ± 39	32 ± 19	22 ± 14
Improved A (3)		292 ± 21	17 ± 14	29 ± 6
Improved B (3)		252 ± 48	22 ± 6	13 ± 5
No change (3)		196 ± 42	36 ± 18	10 ± 9
Pyelonephritis: with hypertension	8	189 ± 30	16 ± 10	14 ± 10
no hypertension	10	192 ± 32	10 ± 7	20 ± 14
Essential hypertension	37	241 ± 30	12 ± 8	12 ± 8
Grade o (7)		249 ± 27	5 ± 2	14 ± 11
Grade I (10)		238 ± 40	9 ± 6	9 ± 7
Grade II (10)		251 ± 29	8 ± 3	10 ± 5
Grade III (10)		230 ± 26	25 ± 14	18 ± 11
Malignant hypertension	10	243 ± 27	4 ± 4	9 ± 6
Pheochromocytoma	7	240 ± 31	7 ± 5	18 ± 10
Glomerulonephritis	10	280 ± 37	4 ± 4	18 ± 11

* JGCC = juxtaglomerular cell count (see text).

total counts (JGCC) averaged 192 ± 21 cells for 25 glomeruli, or 7 to 9 cells per unit (Table I and Fig. 1).

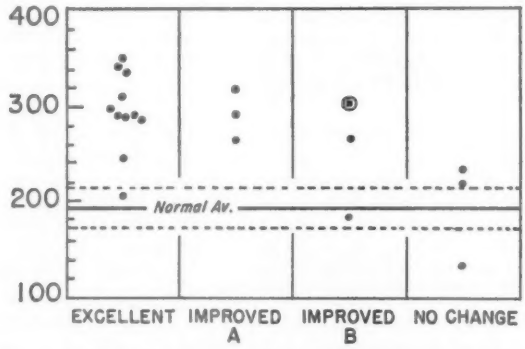
Unilateral Kidney Disease

In the "Goldblatt type" kidneys the average JGCC was 273 ± 53, or 9 to 13 cells per unit (Fig. 3). There were wide variations between the cases, however, and a review of the clinical records revealed interesting relationships between the counts and the patients' clinical courses. First, there was an inverse correlation between the JGCC in most instances and the duration of the hypertension before operation (Text-fig. 1). Second,



TEXT-FIGURE 1. Graphic representation of the relation between the total number of juxtaglomerular cells counted in 25 juxtaglomerular bodies (hatched bars) and the preoperative duration of hypertension (black bars) in 13 cases of primary renal hypertension. The JGCC declined in patients with so-called "Goldblatt" kidneys when the associated hypertension was of longer duration.

the therapeutic effect of the nephrectomy was considered (Text-fig. 2) and classified as follows: (a) Excellent, blood pressure reduced postoperatively to 140/90 mm. of Hg or less for at least one year; (b) improved A, blood pressure usually 140/90 but somewhat higher at times; (c) improved B, blood pressure reduced but still above normal levels; (d) "no change."



TEXT-FIGURE 2. Relationship between the total juxtaglomerular cell count (JGCC) and the therapeutic effectiveness of a unilateral nephrectomy for hypertension. The clinical results were better when the JGCC was significantly increased. The encircled value refers to the 69-year-old woman mentioned in the text.

The patients who were cured or markedly improved were found to have significantly higher cell counts than the others. The one exception was a patient with a high JGCC whose hypertension was reduced, but still remained above normal, i.e., improved B. This patient was 69 years old, had generalized arteriosclerosis and a recent renal arterial thrombosis with ischemia in an already atrophic kidney.

The patients who were cured proved, in general, to be younger than those who did not respond to surgical treatment. Weight determinations of the kidneys, when available, showed that the higher cell counts were found in kidneys with near normal weights (103 ± 31 gm.) while lower counts were noted in atrophic kidneys (71 ± 17 gm.; $p = 0.05$). The group with the poorest therapeutic response had severe arteriosclerosis with involvement not only of a renal artery but of the aorta and of the iliac arteries as well. In these cases, there were also old organized renal infarcts. The cured individuals had either congenital anomalies, thrombosis, endarteritis, external compression or focal sclerosis of one renal artery. In summary, high cell counts in the "Goldblatt type" kidneys were correlated with more acute lesions, a shorter duration of the hypertension, a younger age group, and a favorable prognosis for the relief of hypertension after nephrectomy.

With regard to specific cell types, those with moderately or heavily granulated cytoplasm (types II and III) were increased in all of the "Goldblatt type" hypertensive individuals, as compared to the controls, but this increase was not significant in any particular group. The clear watery cells were decreased in number in the "Goldblatt type" group with the poorest therapeutic response, although this decrease was not quite statistically significant.

As noted before, specimens were obtained from the contralateral kidneys in 5 of the "Goldblatt type" cases (Table II). Total cell counts were lower, and the number of both granulated and clear cells was reduced significantly in the biopsy, in comparison with the affected kidney in the same patient.

Pyelonephritis

Juxtaglomerular cell counts in pyelonephritic kidneys with and without hypertension did not disclose any significant difference from the controls, either in the total number or in the different types of cells (Fig. 2).

Essential Hypertension

Studies of renal specimens in the so-called essential hypertensive group gave unexpected results. The cells of the juxtaglomerular body were significantly increased in number in comparison with the normal aver-

TABLE II

JUXTAGLOMERULAR CELL TYPES AND COUNTS IN 5 "GOLDBLATT TYPE" KIDNEYS WITH BIOPSY OF THE CONTRALATERAL KIDNEY

	JGCC * (total)	Types of cells	
		II and III	IV
"Goldblatt kidney"	290 ± 55	19 ± 9	22 ± 14
Contralateral kidney	191 ± 22	8 ± 3	7 ± 6
	$p < 0.02$	$p < 0.05$	$p < 0.05$

* JGCC = juxtaglomerular cell count.

age, regardless of the degree of arteriolar sclerosis present (Fig. 4). This increase was, however, less marked than in the acute hypertension associated with the "Goldblatt type" kidney ($p < 0.01$). Granularity was increased only in the presence of severe arteriolar sclerosis (grade III), as compared to kidneys without vascular lesions (grade 0) from the cases of essential hypertension. The patients had had hypertension for periods ranging from 2 to 24 years. In contrast with the "Goldblatt type" kidneys, the highest counts were often found in persons who had been hypertensive for long periods of time. Particularly, the number of

clear cells was significantly increased in specimens from patients hypertensive for more than 5 years, as compared to the remainder ($p < 0.05$). No correlation was found between the JGCC and the level of the blood pressure in these patients.

The total JGCC in "malignant" hypertension was in the same range as in benign essential hypertension. A remarkable feature, however, was that the cells were consistently relatively small (Fig. 5). The number of clear cells was significantly below the normal average ($p < 0.05$). According to the available clinical data, these patients had, in general, a terminal malignant phase superimposed on benign essential hypertension of varying duration.

Pheochromocytoma

Seven biopsy specimens obtained during an operative procedure for removal of a pheochromocytoma also showed a significantly increased number of juxtaglomerular cells, similar to that found in essential hypertension (Fig. 6). These patients had had typical paroxysmal hypertension for periods varying from 5 months to 3 years. Assays of 4 pheochromocytomas for catechols were positive.

Glomerulonephritis

The last group, represented by 10 cases of subacute and chronic glomerulonephritis, showed markedly elevated counts on the average. There were fairly wide variations among the cases, however. Patients with symptoms for less than 2 years, or with histologic alterations of subacute glomerulonephritis, had significantly higher counts than the others, whether or not hypertension had developed (Fig. 7). The juxtaglomerular bodies appeared shrunken in kidneys extensively damaged by chronic glomerulonephritis.

Discussion

The most distinctive alteration observed in the juxtaglomerular body in surgical tissues was the significant cellular hyperplasia in cases of hypertension that followed some occlusive process in one renal artery and represented typical examples of primary renal hypertension. This was most striking in younger patients, presumably without other serious vascular lesions, with only slight to moderate renal atrophy, and with hypertension of short duration. These characteristics are regarded clinically as good indications for unilateral nephrectomy, which is often followed by a cure of the hypertension.[15] The hyperplastic aspect of the juxtaglomerular body in a renal biopsy may have an important diagnostic and prognostic value in such cases. Specimens from the contra-

lateral kidney, when available, showed that both the total counts and the types of cells believed to be functionally more active were significantly decreased in the unaffected kidney.

These observations favor the possibility of an endocrine function in the juxtaglomerular cells. These could secrete some hypertensive factor like renin, either following ischemia or, more probably, in response to some hemodynamic change within the kidney.[2,16] Hyperplasia ordinarily indicates increased function in many other secretory cells of the body.

No mitotic figures were observed in the juxtaglomerular cells. The latter may originate by transformation of muscle elements in the afferent arteriole into secretory cells.[1,7] In most instances, an analogous transformation of the muscle components in afferent or even interlobular arterioles was noted to accompany the hypertrophy of the juxtaglomerular cells. These muscle cells may enlarge, acquire watery cytoplasm (Fig. 3), and even come to contain Bowie-positive granules; their behavior thus appears closely related to that of the juxtaglomerular body.

The clear watery cells (type IV) presumably reflected an excess in both hormone production and secretion. It should be noted that the granularity of the juxtaglomerular cells in man is much less pronounced than in animals. The increase in the number of human cells was in general more significant and reliable than modifications in their granularity. A similar phenomenon has also been observed in certain animals,[6] and there may be a definite species difference in man, precluding biologic generalizations or sweeping conclusions. As can be seen in Table I, individual variations in granularity were wide, but the deviations appeared significant only in the "Goldblatt type" cases and in essential hypertension with grade III arteriolar sclerosis.

The stains now available for juxtaglomerular granules are selective but not specific, and the nature of the granules is unknown. They may represent stored vasopressor substance or a precursor of the active proteolytic enzyme, renin. A close correlation between renin extracted from the kidney and the number of granules in the juxtaglomerular cells has been shown in animals.[17] Analogous results in human subjects were not obtained in our series, perhaps because of technical difficulties.[18] Hartroft and Edelman [19] recently demonstrated, in rabbits and rats, a selective absorption by the juxtaglomerular granules of fluorescent antibodies to almost pure renin.

These results are highly suggestive of the presence of a vasopressor substance like renin or of a precursor in the juxtaglomerular cells. Proteolytic enzymes seldom exist as such intracellularly, and some autolysis

is necessary before renin can be extracted biochemically from renal tissue.

The problem is still unsolved in cases of pyelonephritis with hypertension. No definite juxtaglomerular alteration was found. The patients with unilateral pyelonephritis, in general, had a moderate hypertension for several years; their average age was 53 ± 15 years, and they were not cured of their hypertension by a nephrectomy. One exception was a 22-year-old woman with a blood pressure of 220/130 for 17 months; removal of the diseased kidney was followed by a return of the blood pressure to normal levels.

In pyelonephritis with hypertension, the arcuate and interlobular arteries have thicker walls and a more reduced lumen than in pyelonephritis without hypertension.[20] The average diastolic pressure is higher in patients with pyelonephritis for the same degrees of arteriolar sclerosis as compared to individuals with essential hypertension.[21] A characteristic proliferative endarteritis with resulting narrowing of the lumen has been repeatedly described in pyelonephritis.[22,23] There may be a prolonged low-grade internal ischemia or hemodynamic modification in the kidney following injury to the vessels in pyelonephritis, with hypertension as a final result. Further investigation, especially in younger patients with early hypertension, would be desirable to prove this hypothesis.

In the examples of essential hypertension investigated, a significant increase in the total number of juxtaglomerular cells was noted. The average number of granular and clear cells did not differ in the various subgroups, except for increased granularity in the group with severe arteriolar sclerosis (grade III). The number of clear cells, however, was found to increase in relation to the duration of the hypertension in individual cases ($p < 0.05$). The transformation of muscle cells in afferent arterioles into round clear or granular cells was particularly striking. Very similar features were observed in the kidney tissues from patients with pheochromocytoma. In the latter groups, the change was very likely secondary to intermittent secretion of catecholamines, spasm and anatomic alterations in the renal vasculature.[24,25] This similarity does not exclude a primary renal vasopressor factor in essential hypertension. On the other hand, secondary changes both in renal vessels[13] and juxtaglomerular cells could contribute renal factors with the property of sustaining early functional hypertension and making it irreversible.

If the large clear cells are presumed to be hyperactive, and the small cells nonfunctioning, the reduced number of clear cells in "malignant" hypertension appears paradoxical. It is possible that a blood pressure high enough to cause arteriolar necrosis may also inhibit the juxtaglo-

merular secretion by a mechanism opposite to that found in "Goldblatt type" kidneys. The increase of hypertensin once reported in such cases [26] would remain unexplained, since renin is thought to be necessary to transform hypertensinogen into hypertensin.

The findings in glomerulonephritis are in agreement with Goormaghtigh's original description, as were those in the "Goldblatt type" kidneys. There was hyperplasia in early stages, regardless of the level of blood pressure, and atrophy in the extensively scarred kidneys. The absence of hypertension in some cases of subacute glomerulonephritis is not presently explainable. It may be necessary to invoke such a factor as a modification in the enzymatic system for degradation of pressor amines to explain the persistent hypertension in patients with severely damaged kidneys and atrophic juxtaglomerular bodies as were found in chronic glomerulonephritis, advanced chronic pyelonephritis and protracted "Goldblatt type" kidney lesions. Recently an early increase in the secretion of renin was reported in experimental renal hypertension. This was followed in the chronic stages by a decreased secretion of renin and a change in the ratio of renal amine-oxidase to dopa-decarboxylase.[27]

Summary

A statistically significant hyperplasia and some increased granularity of renal juxtaglomerular cells occurred in the affected kidney in proved cases of primary renal hypertension in man. These changes were more marked in patients with hypertension for less than one year who were cured or markedly improved by nephrectomy. The observations support Goormaghtigh's original concept of an endocrine function in the juxtaglomerular cells, which could thus secrete some hypertensive factor, probably renin, following an alteration in renal hemodynamics.

The significant juxtaglomerular cell hyperplasia found in the early stages of glomerulonephritis is also considered to constitute a potential cause of primary renal hypertension. A moderate hyperplasia of the juxtaglomerular body in patients with pheochromocytoma, and similar findings in essential hypertension, are thought to be secondary reactions to arteriolar spasm and sclerosis. Relationships were less clear between the structure of the juxtaglomerular body and malignant hypertension or hypertension associated with chronic pyelonephritis.

References

1. Goormaghtigh, N. La Fonction Endocrine des Artérioles Rénales. Son Role dans la Pathogénie de l'Hypertension Artérielle. R. Fonteyne, Louvain, 1944, 110 pp.
2. Allen, A. C. The Kidney; Medical and Surgical Diseases. Grune & Stratton, New York, 1951, pp. 32–34; 396–416.

3. HARTROFT, P. M., and HARTROFT, W. S. Studies on renal juxtaglomerular cells. I. Variations produced by sodium chloride and desoxycorticosterone acetate. *J. Exper. Med.*, 1953, **97**, 415–428.

4. HARTROFT, P. M., and HARTROFT, W. S. Studies on renal juxtaglomerular cells. II. Correlation of the degree of granulation of juxtaglomerular cells with width of the zona glomerulosa of the adrenal cortex. *J. Exper. Med.*, 1955, **102**, 205–212.

5. PITCOCK, J. A., and HARTROFT, P. M. The juxtaglomerular cells in man and their relationship to the level of plasma sodium and to the zona glomerulosa of the adrenal cortex. *Am. J. Path.*, 1958, **34**, 863–883.

6. TOBIAN, L. Physiology of the juxtaglomerular cells. *Ann. Int. Med.*, 1960, **52**, 395–410.

7. KAUFMANN, W. The Goormaghtigh cells in the normal and diseased human kidney; their possible relationship to renal hypertension. *Am. J. Path.*, 1942, **18**, 783–797.

8. DES PREZ, J., JR. The juxtaglomerular apparatus of the hypertensive kidney. *Am. J. Clin. Path.*, 1948, **18**, 953–960.

9. DUNIHUE, F. W. The juxtaglomerular apparatus in experimental hypotension. (Abstract) *Am. J. Path.*, 1947, **23**, 906–907.

10. WIEDMAN, M. L., ROBERTSON, W. VAN B., and DUNIHUE, F. W. The effect of sodium chloride and of mineralo-corticoids on the granulation of juxtaglomerular cells. (Abstract) *Anat. Rec.*, 1956, **124**, 381–382.

11. JUTZLER, G. A. Beitrag zur Morphologie des juxtaglomerulären Apparatus bei Drosselungshockdruck. *Frankfurt. Ztschr. Path.*, 1956, **67**, 177–195.

12. CELORIA, G., GODDARD, J. W., and SOMMERS, S. C. Unpublished observations.

13. SOMMERS, S. C.; RELMAN, A. S., and SMITHWICK, R. H. Histologic studies of kidney biopsy specimens from patients with hypertension. *Am. J. Path.*, 1958, **34**, 685–715.

14. WILSON, W. A new staining method for demonstrating the granules of the juxtaglomerular complex. *Anat. Rec.*, 1952, **112**, 497–507.

15. PERERA, G. A., and HAELIG, A. W. Clinical characteristics of hypertension associated with unilateral renal disease. *Circulation*, 1952, **6**, 549–552.

16. TOBIAN, L.; TOMBOULIAN, A., and JANECEK, J. The effect of high perfusion pressures on the granulation of juxtaglomerular cells in an isolated kidney. *J. Clin. Invest.*, 1959, **38**, 605–610.

17. TOBIAN, L. A.; JANECEK, J., and TOMBOULIAN, A. Correlation between granulation of juxtaglomerular cells and extractable renin in rats with experimental hypertension. *Proc. Soc. Exper. Biol. & Med.*, 1959, **100**, 94–96.

18. HAAS, E., and GOLDBLATT, H. Renin content of kidneys in experimental renal and human essential hypertension. *Am. J. Physiol.*, 1959, **197**, 1103–1106.

19. HARTROFT, P. M., and EDELMAN, R. Renal Juxtaglomerular Cells in Sodium Deficiency. Effects of Hypophysectomy; Localization of Renin by the Fluorescent Antibody Technique. In: Edema, Mechanisms and Management. A Hahnemann Symposium on Salt and Water Retention. MOYER, J. H., and FUCHS, M. (eds.) W. B. Saunders Co., Philadelphia, 1960, pp. 63–68.

20. ROBBINS, G. B.; BABIN, D. S.; TURGEON, C., and SOMMERS, S. C. Kidney maps in the study of renal disease. (In preparation.)

21. MERRIAM, J. C.; SOMMERS, S. C., and SMITHWICK, R. H. Clinicopathologic correlations of renal biopsies in hypertension with pyelonephritis. *Circulation*, 1958, **17**, 243–248.

22. Weiss, S., and Parker, F., Jr. Pyelonephritis; its relation to vascular lesions and to arterial hypertension. *Medicine,* 1939, **18,** 221–315.

23. Kincaid-Smith, P. Vascular obstruction in chronic pyelonephritic kidneys and its relation to hypertension. *Lancet,* 1955, **2,** 1263–1268.

24. Moyer, J. H., and Handley, C. A. Norepinephrine and epinephrine effect on renal hemodynamics, with particular reference to the possibility of vascular shunting and decreasing the active glomeruli. *Circulation,* 1952, **5,** 91–97.

25. Silva, T. F., and Sommers, S. C. Renal biopsy changes with pheochromocytoma. *Am. J. M. Sc.,* 1958, **236,** 700–704.

26. Kahn, J. R.; Skeggs, L. T., Jr.; Shumway, N. P., and Wisenbaugh, P. E. The assay of hypertensin from the arterial blood of normotensive and hypertensive human beings. *J. Exper. Med.,* 1952, **95,** 523–529.

27. Giordano, C.; Samiy, A. H.; Bloom, J.; Haynes, F. W., and Merrill, J. P. Studies on experimental renal hypertension in rabbits. (Abstract) *Fed. Proc.,* 1960, **19,** 100.

[*Illustrations follow*]

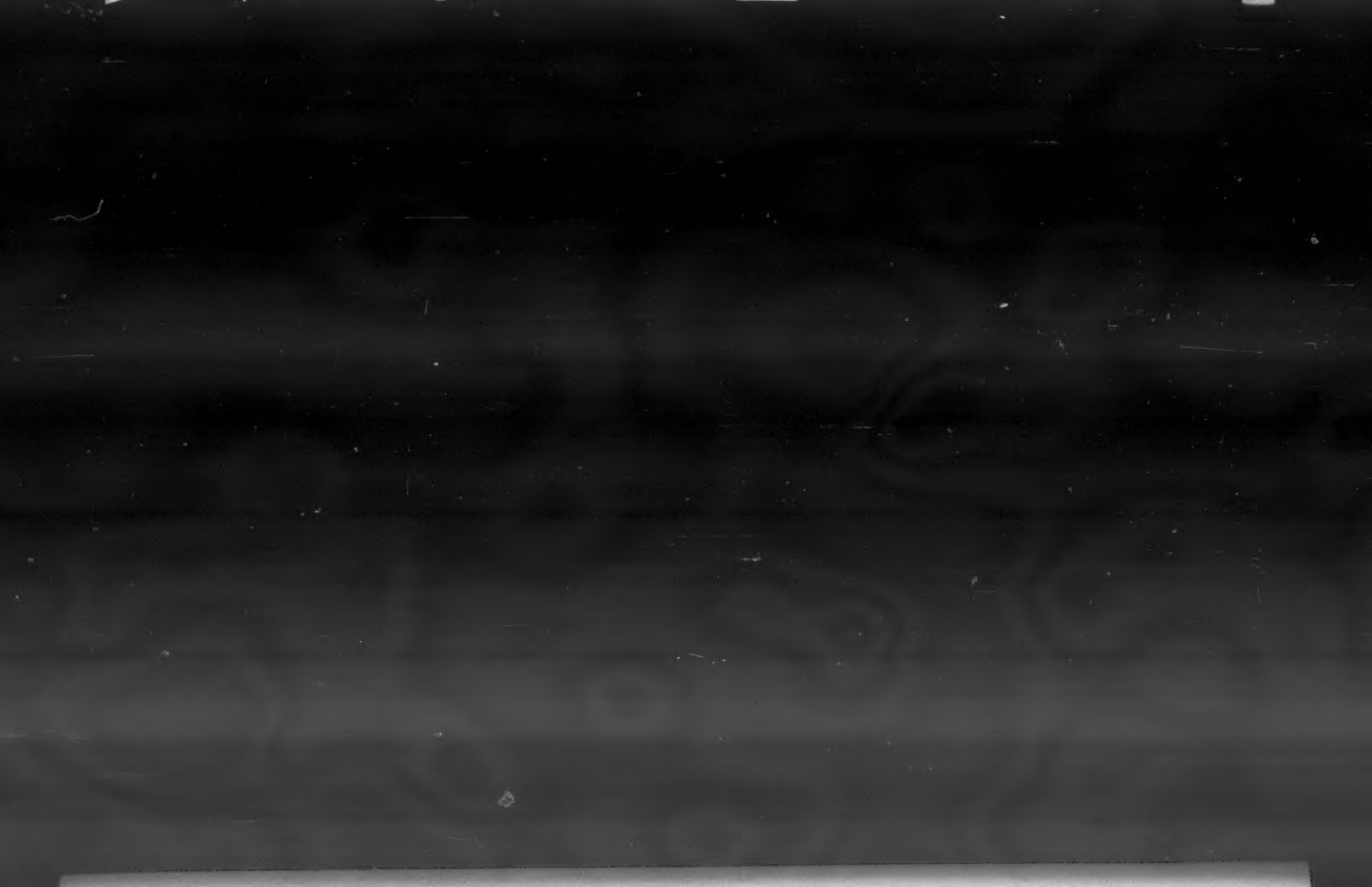
Legends for Figures

All illustrations were prepared from tissues stained by the Bowie method.[8]

Fig. 1. Juxtaglomerular body, kidney of a 49-year-old man without hypertension, operated upon for renal carcinoma. The dark fine cytoplasmic granules in the juxtaglomerular cells were deep purple with the Bowie stain. × 450.

Fig. 2. Kidney with nephrolithiasis and severe chronic pyelonephritis; 32-year-old woman with hypertension. Bowie-stained cytoplasmic granules are evident in some of the convoluted tubular epithelium. Juxtaglomerular cells are shown near the bottom of the illustration. × 450.

Fig. 3. Kidney with localized endarteritis and narrowing of the renal artery; 21-year-old man with hypertension of short duration. The clinical result following nephrectomy was excellent, with relief of the hypertension. A hyperplastic juxtaglomerular apparatus, with an increased number of enlarged hydropic (type IV) juxtaglomerular cells and a similar transformation of some cells in the afferent arteriole. × 240.



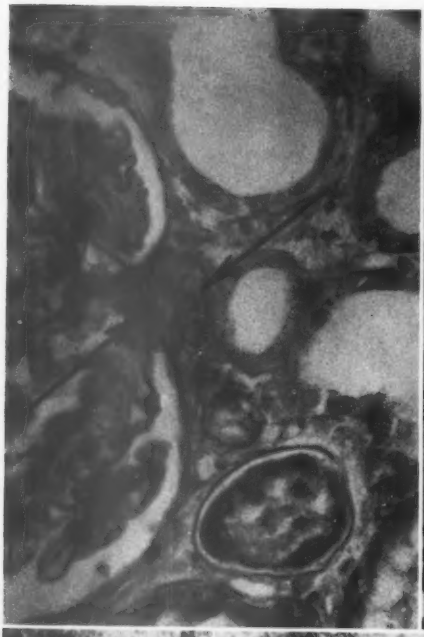


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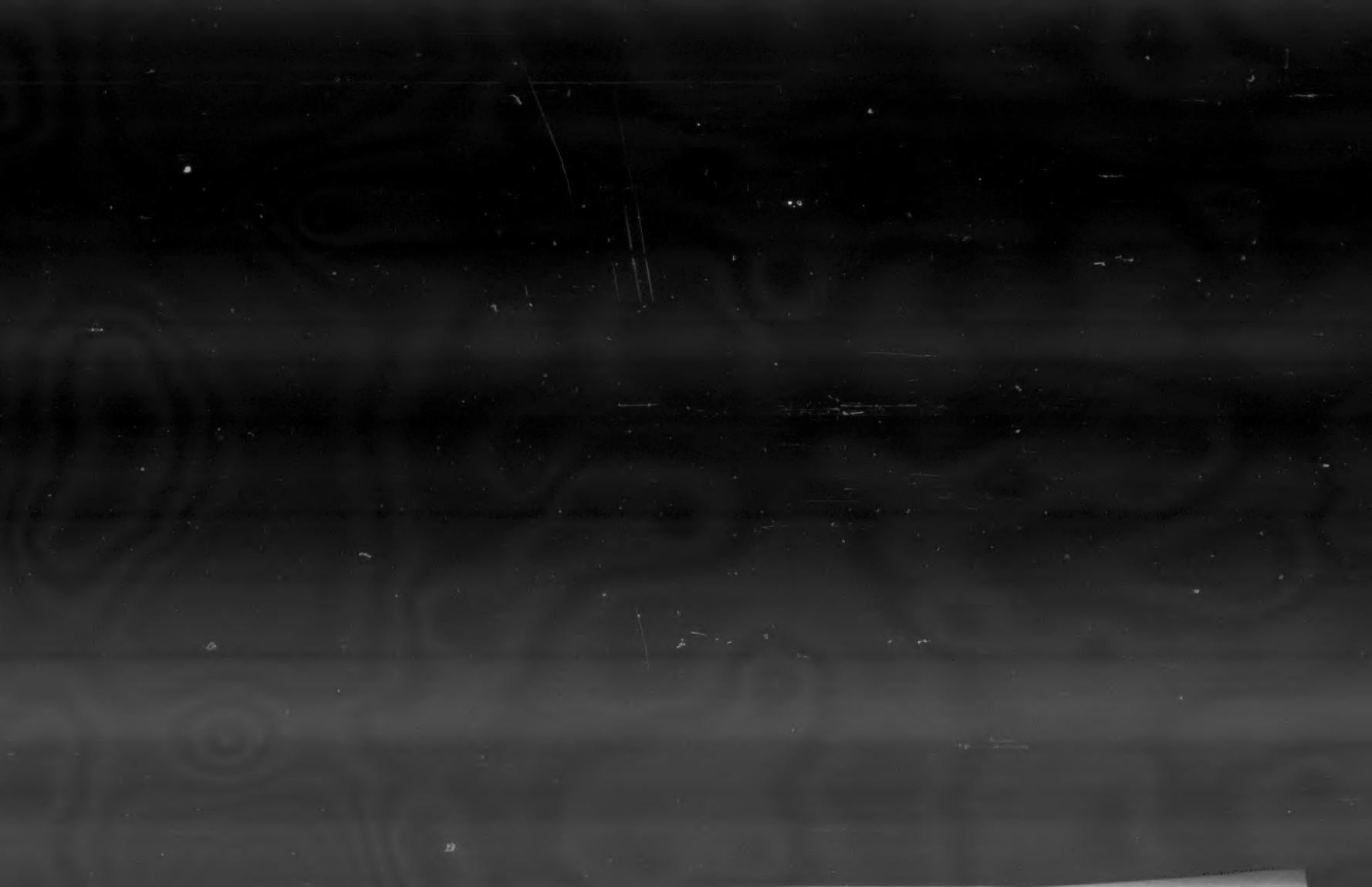
FIG. 4. A renal biopsy taken at sympathectomy from a patient with essential hypertension and moderate arteriolar nephrosclerosis (grade II). There is hyperplasia of a juxtaglomerular body which includes several type IV cells. Bowie-positive granules are present in the tubular epithelium. × 450.

FIG. 5. Shrunken juxtaglomerular cells in malignant hypertension with severe arteriolar nephrosclerosis (grade III). The dark smudges represent focal fibrinoid necrosis in the afferent arteriole and glomerulus. × 450.

FIG. 6. A renal biopsy taken at operation for removal of a pheochromocytoma. Hyperplasia of the juxtaglomerular cells and a cross section of an unaltered afferent arteriole are demonstrated. × 450.

FIG. 7. Juxtaglomerular apparatus in a case of chronic glomerulonephritis. The hyperplastic changes evident were not correlated with hypertension. × 450.



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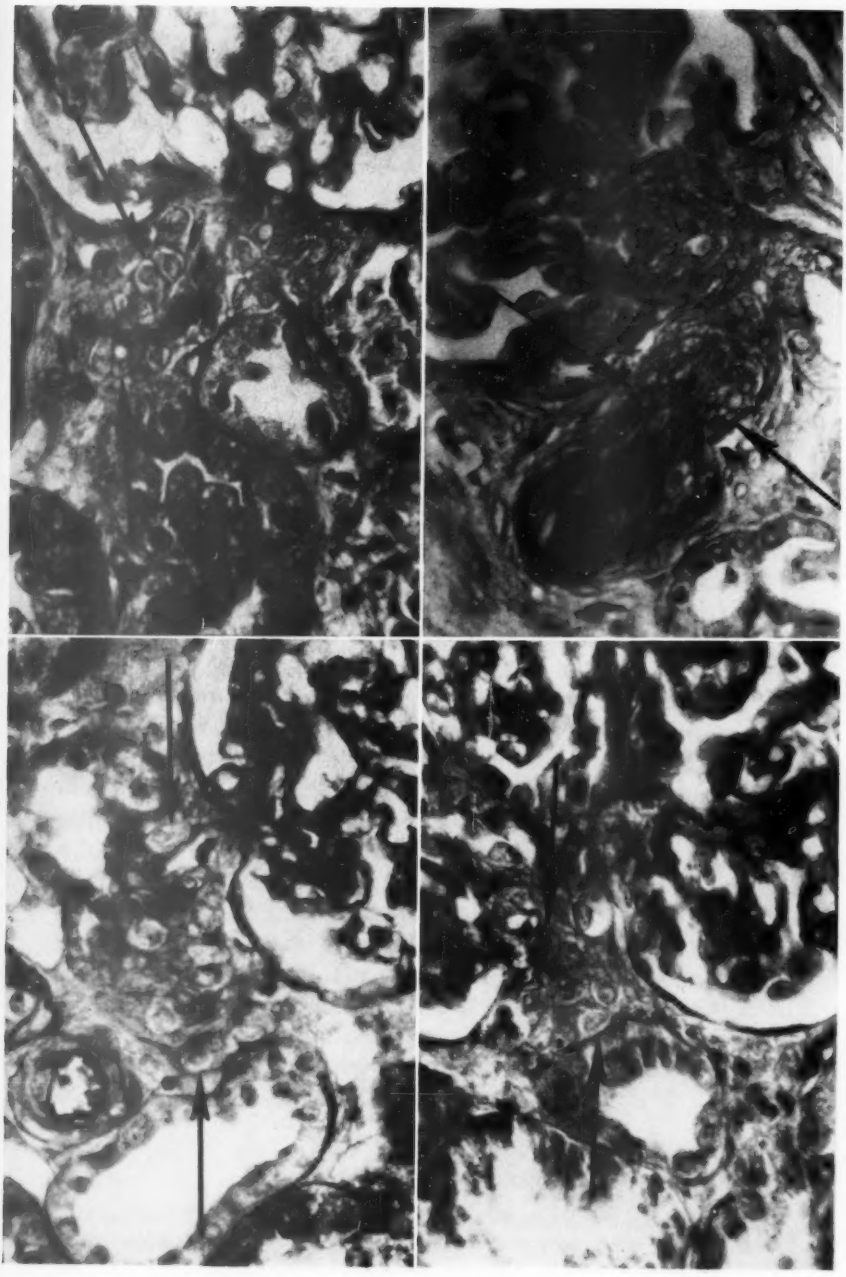

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PANETH CELL METAPLASIA IN ULCERATIVE COLITIS

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The pathologic diagnosis of ulcerative colitis is reached by exclusion. In the absence of specific infective agents or lesions, the pathologist depends principally upon the clinical history, the characteristic gross appearance of the ulcers, and the striking tendency for pseudopolyp formation. However, the identification of certain microscopic changes may be helpful in many cases, notably the presence of cryptitis, nonspecific ulceration, focal granulomas, etc. We have recently become interested in another microscopic alteration—Paneth cell metaplasia—a lesion which was described by Hertzog[1] in a single case of ulcerative colitis 13 years ago but which apparently has not been mentioned in any modern treatise on the pathology of the disease.[2-7]

Paneth cells are normally present in the mucosa of the small intestine, but most authorities state that they are absent from the normal colon.[8,9] Nevertheless, it should be noted that Bloch[10] found numbers of these cells in the large intestine of newborn infants although they could not usually be demonstrated at older ages. However, he did find Paneth cells in one adult with appendicitis, and Hertzog[1] noted them in 3 of 25 diseased colons—in the case of ulcerative colitis mentioned above and in two cases of tuberculous typhlitis. Our interest in these cells stems from an accidental observation made during the microscopic study of a segment of colon removed surgically from an individual with long-standing ulcerative colitis. On preliminary examination the impression was gained that Paneth cells were almost as numerous in the colonic crypts as they were in the mucosa of the accompanying segment of terminal ileum (Fig. 1). The metaplastic change was so striking that it stimulated us to search for it in tissue from other surgically treated cases of ulcerative colitis in our hospital and in an associated institution. The results of this review and of the observations in a comparable number of colons resected for other diseases are the basis of the present report.

Material and Method

Tissue was obtained from all cases of ulcerative colitis that had been treated by partial colectomy in our hospital over a period of 10 years. Since only 4 such cases

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were available, the series was augmented by 6 additional specimens from cases of ulcerative colitis treated surgically at Victoria Hospital, London, Ontario. Multiple sections of the colon were cut at intervals from the paraffin blocks already available from each of these 10 cases; they were stained with hematoxylin and eosin and examined microscopically for the presence and the incidence of Paneth cells. The exact procedure was to count the number of glands in as many low power (× 70) microscopic fields as possible, the maximum number of microscopic fields counted in any one case being 100. The number of glands containing one or more Paneth cells was then noted. To avoid counting the same gland twice in any one microscopic field, only the bases of the crypts were considered; similarly, the only Paneth cells counted were those at the bases of the same crypts. The criterion for the diagnosis of Paneth cells was the presence of large eosinophilic granules, supranuclear in position, lying in the cytoplasm of cells at the bases of the crypts[9] (Fig. 2).

Similarly, the incidence of Paneth cells was determined in colons which had been resected for diseases other than ulcerative colitis. Ten such specimens were obtained from our own files or from Victoria Hospital. Most of them were examples of partial colectomy performed for adenocarcinoma. The search for Paneth cells in this material was made from blocks of tissue as far distant as possible from the sites of neoplasm.

No attempt has been made to determine the incidence of Paneth cell metaplasia of the colon in necropsy material as it was suspected that postmortem autolysis might interfere with the staining qualities of the specific cytoplasmic granules.[3]

Observations

Each of the 10 cases listed in Table I fulfilled the criteria described for ulcerative colitis, both pathologically and clinically. They have been listed in the table in the order of duration of symptoms. The percentages of glands showing the presence of one or more Paneth cells at the bases of crypts in each case of ulcerative colitis are given in the same table. It will be noted that the number of crypts showing the presence of these cells varied from 0.6 per cent to 30 per cent (average 19.6 per cent) and that, with the exception of case 8, there was a reasonable correlation between the incidence of Paneth cells and the clinical duration of disease. This is in marked contrast to the number of Paneth cells found at the bases of crypts in the control specimens (Table II), in which the average number was only 0.3 per cent.

The number of Paneth cells found in any one crypt in the cases of ulcerative colitis varied considerably; generally, 1 or 2 of these cells were found although occasionally as many as 5 could be distinguished. In addition, a diminution in the number of crypts per low power field, compared with that in the control group, was present in the ulcerative colitis. This has been noted previously by Lumb.[5]

No Paneth cells were seen adjacent to areas of recent acute ulceration unless evidence of previous damage was present; nor were their granules ever noted within a crypt abscess. The cells were usually found in areas which were interpreted as regenerated or previously damaged mucosa. In these locations the crypts were often irregular, the mucosa thinner

than normal, and the submucosa scarred. In one instance, Paneth cells were seen in the depth of a crypt which was, or appeared to be, affected by cryptitis at a different level (Fig. 3); and in another, a small clump of cells which contained a few eosinophilic granules lay at the margin of a micro-abscess. These latter findings, although suggestive of an association between Paneth cells and cryptitis, were by no means definite.

TABLE I

THE INCIDENCE OF PANETH CELL METAPLASIA OF THE COLON IN ULCERATIVE COLITIS

Case no.	Age (yr.)	Sex	Duration of disease (yr.)	No. of low-power fields counted	Total crypts counted	Av. no. of crypts per low-power field	Total no. of crypts with one or more Paneth cells	Percentage of crypts with one or more Paneth cells
1	47	F	20	43	245	6	63	26.0
2	37	M	17	100	649	6	174	27.0
3	39	F	16	94	585	6	174	30.0
4	32	M	13	100	935	9	252	27.0
5	49	M	10	100	928	9	243	26.0
6	29	M	9	100	682	7	124	18.0
7	20	F	4	38	255	7	31	12.0
8	21	M	1½	98	780	8	191	24.0
9	20	F	1½	52	418	8	20	5.0
10	36	F	1	55	311	6	2	0.6
							Average	19.6

TABLE II

THE INCIDENCE OF PANETH CELL METAPLASIA IN "NORMAL" COLONS

Case no.	Age (yr.)	Sex	No. of low-power fields counted	Total crypts counted	Av. no. of crypts per low-power field	Total no. of crypts with one or more Paneth cells	Percentage of crypts with one or more Paneth cells
11	72	M	100	1114	11	3	0.3
12	76	M	100	1407	14	2	0.1
13	57	M	100	1102	11	3	0.3
14	63	M	100	1348	13	8	0.6
15	68	M	100	1234	12	2	0.2
16	80	F	100	1187	12	1	0.1
17	77	F	100	979	9	14	1.4
18	74	F	100	1182	12	0	0.0
19	65	M	100	1125	11	4	0.4
20	45	M	100	1320	13	0	0.0
						Average	0.3

DISCUSSION

Contrary to the views expressed in earlier reports,[1,8,9] our results indicate clearly that Paneth cells are present in the mucosal crypts of most "normal" colons. However, their numbers are so sparse that they can only be demonstrated by a searching examination of many microscopic fields in a particular specimen. On the other hand, their number

has been found to be enormously increased (even as much as 300 fold) in association with the regenerative phase of ulcerative colitis (Tables I and II).

The obvious question arises: Are such large numbers of Paneth cells in the colon of any importance in the initiation of ulcerative colitis? We doubt if this is so because of the definite suggestion in our cases that the more long-standing the disease process, the greater the number of Paneth cells (Table I). Indeed, we found very few of them in the colon from one individual (case 10) who had suffered from symptoms for only one year. This being the case, the presence of Paneth cells in association with long-standing ulcerative colitis must be interpreted as a metaplastic alteration—as the differentiation of previously undifferentiated cells at the bases of colonic crypts. They are therefore regarded as the result, rather than the cause, of colonic inflammation or regeneration. This is analogous to the intestinalization of gastric mucosa found in chronic gastritis. This interpretation, however, does not necessarily mean that Paneth cells in these locations have no pathogenetic significance; it does not eliminate the possibility that they may assist in perpetuating the disease.

The exact function of the Paneth cells in man is still a mystery. At one time they were presumed to secrete the ferments of the intestinal juice,[11] and this view has had a certain amount of support in recent publications. For example, Blaschko and Jacobson,[12] citing van Weel, asserted that Paneth cells in the ileum of the rat produced a proteolytic exoenzyme, dipeptidase. Marques de Castro, Sasso and Saad[13] also presented evidence that the secretion of these cells in the ant eater was capable of digesting chitin, whereas in the rabbit it digested scleroprotein. If it can be shown that the Paneth cells of man have similar or related proteolytic actions, their liberation into the lumens of colonic crypts offers a possible explanation for the production of micro-abscesses (cryptitis) and for the spreading mucosal inflammation which are such characteristic features of the disease.[4,6] This hypothetical mechanism should apply particularly in patients in remission, since with normal dehydration of the fecal stream there would be less dilution of the liberated ferments and more chance of a local histolytic effect. Further support to this suggestion is found in the observation that even surgically defunctioned colons were subject to continuing active inflammation and ulceration.[4] The words of Warren and Sommers[6] are also pertinent: "Presence of early lesions [cryptitis] in a uniform location within the crypts, even though scattered along the course of the intestine, suggests . . . that the irritant is being released by the mucosa. The possible role of such agents as proteolytic enzymes in ulcerative colitis already has

aroused much clinical interest, but their origin from the colonic mucosa has not been considered previously and now requires further investigation."

More information on the functions of Paneth cells is therefore required, and in particular on the possible proteolytic action of their granules. Until this information is available, the significance of Paneth cell metaplasia in the pathogenesis of ulcerative colitis must remain speculative. This is particularly so since we have not yet succeeded in demonstrating, unequivocally, Paneth cells in intimate association with a micro-abscess in a single colonic crypt.

Summary

A searching examination of many microscopic fields of colons resected surgically for ulcerative colitis, and for other diseases, has revealed Paneth cells in the colonic crypts of most of the specimens. They were exceedingly sparse in "normal" colons, but their numbers were markedly increased in cases of ulcerative colitis (as much as 300 fold). The widespread Paneth cell metaplasia of the large intestine is considered to be the result, rather than the cause, of ulcerative colitis. On the other hand, the possibility that Paneth cells in the colonic crypts may be important perpetuating agents of the disease has not been eliminated.

References

1. Hertzog, A. J. The Paneth cell. *Am. J. Path.*, 1937, **13**, 351–360.
2. Goldgraber, M. B.; Kirsner, J. B., and Palmer, W. L. The histopathology of chronic ulcerative colitis and its pathogenic implications. *Gastroenterology*, 1960, **38**, 596–604.
3. Lumb, G., and Protheroe, R. H. The early lesions of ulcerative colitis. *Gastroenterology*, 1957, **33**, 457–474.
4. Lumb, G., and Protheroe, R. H. B. Ulcerative colitis; a pathologic study of 152 surgical specimens. *Gastroenterology*, 1958, **34**, 381–407.
5. Lumb, G., and Protheroe, R. H. B. Biopsy of the rectum in ulcerative colitis. *Lancet*, 1955, **2**, 1208–1215.
6. Warren, S., and Sommers, S. C. Pathogenesis of ulcerative colitis. *Am. J. Path.*, 1949, **25**, 657–679.
7. Watkinson, G.; Thompson, H., and Goligher, J. C. Right-sided or segmental ulcerative colitis. *Brit. J. Surg.*, 1960, **47**, 337–351.
8. Ham, A. W. Histology. J. B. Lippincott, Philadelphia, 1957, ed. 3, 894 pp.
9. Macklin, C. C., and Macklin, M. T. The Intestinal Epithelium. In: Special Cytology. The Form and Functions of the Cell in Health and Disease. Cowdry, E. V. (ed.) Paul B. Hoeber, Inc., New York, 1932, ed. 2, Vol. 1, p. 264.
10. Bloch, C. E. Anatomische Untersuchungen über des Magendarmkanal des Säuglings. *Jahrb. f. Kinderh.*, 1903, **58**, 121–174.
11. Best, C. H., and Taylor, N. B. The Physiological Basis of Medical Practice. Williams & Wilkins Co., Baltimore, 1943, ed. 3, 1942 pp.

12. BLASCHKO, H., and JACOBSON, W. Enzyme Systems of Cells. In: Cytology and Cell Physiology. BOURNE, G. H. (ed.) Oxford University Press, London, 1951, ed. 2, Chapt. 8, pp. 322–372. (Citing Van Weel, P. B. Beiträge zur Histophysiologie des Dünndarmes; die Veränderungen in den Darmdrüsenzellen während der Verdauung und in den Resorptionszellen während der Fettresorption. *Ztschr. Zellforsch.*, 1939, **29**, 750–771.)

13. MARQUES DE CASTRO, N.; SASSO, W. DA S., and SAAD, F. A. Preliminary observations of the Paneth cells of the *Tamandua tetradactyla Lin. Acta anat.*, 1959, **38**, 345–352.

We are indebted to Professor J. H. Fisher for supplying us with pathologic specimens from Victoria Hospital, London, Ontario.

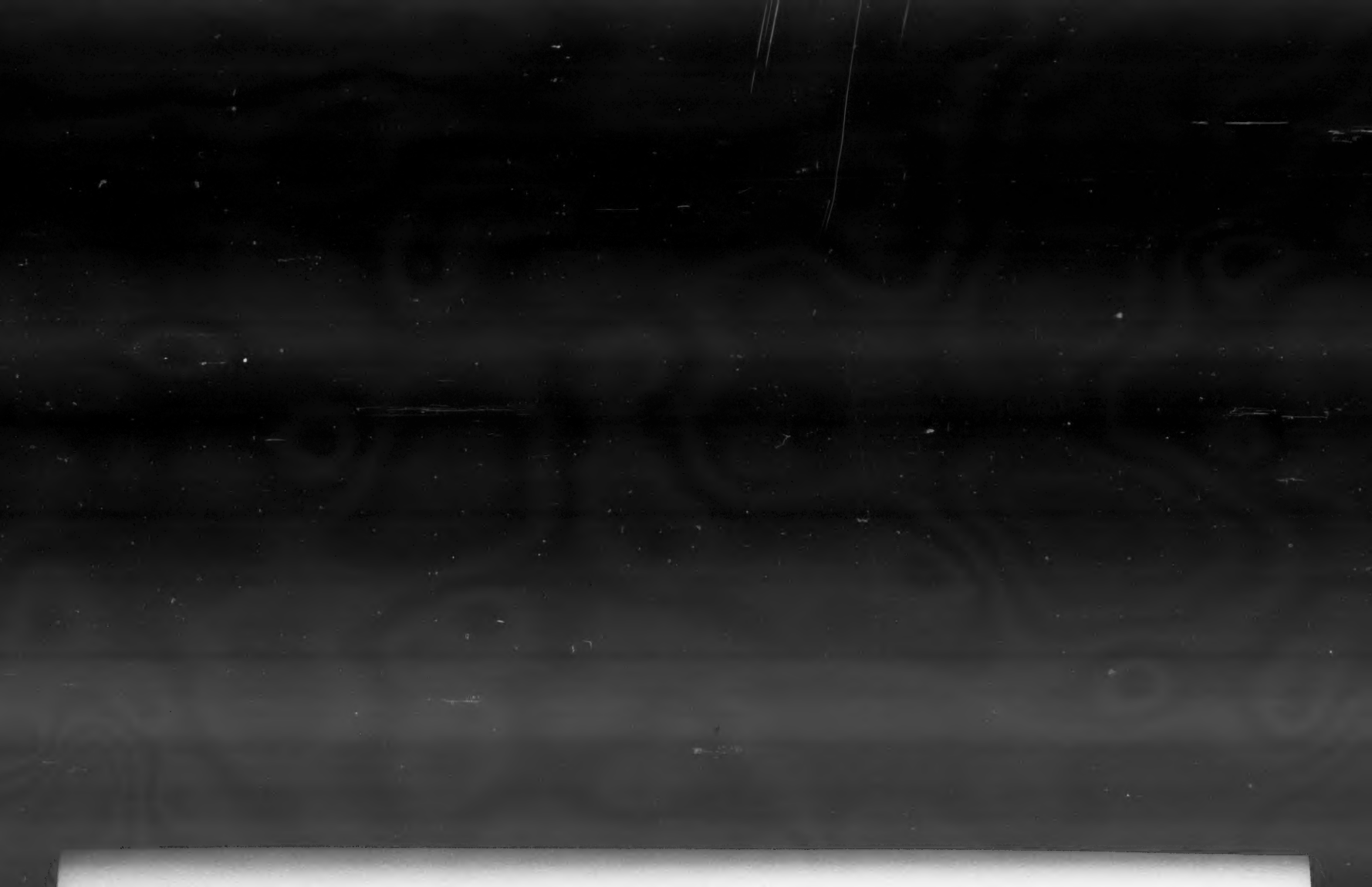
LEGENDS FOR FIGURES

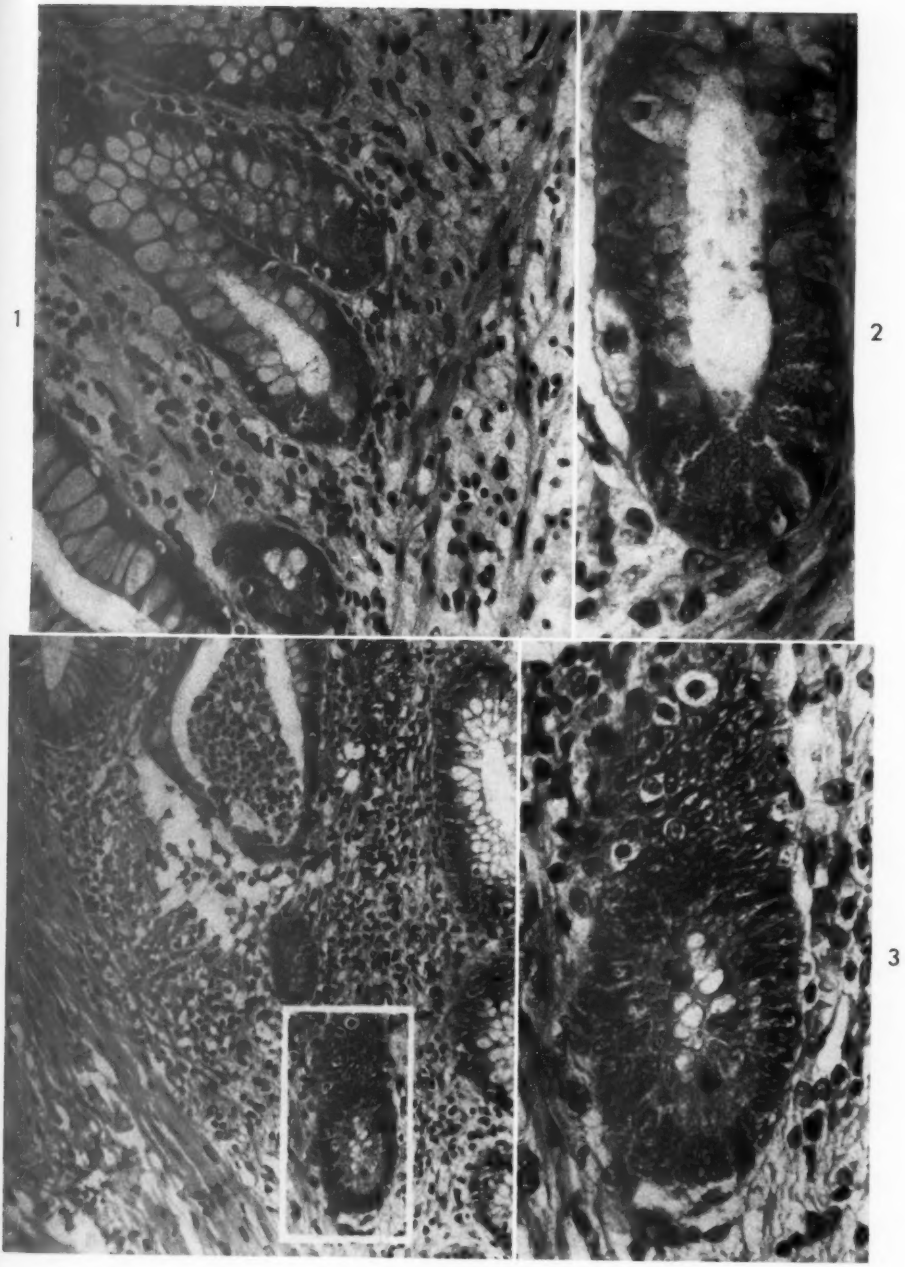
Photographs were prepared from sections stained with hematoxylin and eosin.

FIG. 1. Four crypts in the large intestine of a case of long-standing ulcerative colitis (case 5). Each crypt has numbers of Paneth cells at its base. × 300.

FIG. 2. A single colonic crypt containing numerous Paneth cell granules, supranuclear in position, at the base of the crypt. × 600.

FIG. 3. The crypt of Lieberkühn on the left contains a micro-abscess at one level. At the base of the crypt, shown in the box and magnified on the right, there are several Paneth cells. Left, × 200; right, × 600.





RHEUMATIC PHLEBITIS OF THE CORONARY VEINS

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In the older literature are found many references to rheumatic phlebitis, usually with thrombosis. The vessels most frequently involved were in the upper part of the body. The changes in the veins were those associated with an organizing thrombus, and it is doubtful that with our present knowledge of the lesions of rheumatic fever, any of these reported cases would be accepted as being of rheumatic origin. The pertinent publications have been summarized by Sladen and Winternitz[1] and need not be reviewed here. There are few reports of rheumatic phlebitis in recent publications. Pappenheimer and Von Glahn[2] mentioned the involvement of a myocardial vein in a case of rheumatic fever. The vein wall was lifted into irregular prominences by a mass of cells that replaced the muscular elements and pushed the intact overlying endothelium into the lumen. In the legends accompanying the illustrations of this lesion, it is stated that the vein wall was infiltrated with neutrophils and large basophilic cells with distorted nuclei. The endothelium was swollen.

Shaw[3] described an acute vascular lesion affecting the venules and, less frequently, arterioles in cases of rheumatic fever. The vessels affected were situated in the myocardium, auriculoventricular sulcus, the connective tissue between the auricles and atrial trunks, and the mediastinum. All coats of the vessels were infiltrated with many neutrophils. The intact endothelial lining was raised like a blister over the cellular exudate. Usually only a sector of the vessel wall was involved, rarely the whole circumference. Absence of thrombosis was a striking feature although specific stains disclosed an abundance of fibrin beneath the endothelium and extending outward into the adventitia. Gräff,[4] in a discussion of the changes in the tonsil, mentions an infiltration of the wall of a vein that he believed to be of rheumatic origin and that was accompanied by thrombosis. Perry[5] reported phlebitis with thrombosis in a young girl who had had two attacks of rheumatic fever. The left subclavian, internal jugular and innominate veins and the right internal jugular vein were occluded by thrombi. The adventitia of the veins was hyperemic and infiltrated with small round cells. Loose collections of

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TABLE I

OCCURRENCE OF LESIONS OF CORONARY SINUS AND CORONARY VEINS IN 18 CASES OF ACTIVE RHEUMATIC HEART DISEASE

Case no.	Age	Duration of clinical symptoms	Clinical diagnosis	Sinus lesions * A	B	C	D	Small veins * B	C	D	Other rheumatic lesions
22609	48	Unknown	Carcinoma of ovary								Active endocarditis of mitral and aortic valves.
33351	4	6 wk.	RHD †						+		Active left auriculitis, myocarditis, endocarditis of mitral and aortic valves.
33503	5	6 mo.	RHD			+					Active left auriculitis, myocarditis, endocarditis of mitral and aortic valves, pericarditis.
35092	15	Unknown	RHD	+	+	+	+	+	+		Active right and left auriculitis, myocarditis, endocarditis of mitral, aortic and tricuspid valves, pericarditis.
35296	4	4 mo.	RHD		+	+					Active left auriculitis, myocarditis, endocarditis of tricuspid and mitral valves, pericarditis.
37034	7	2 yr.	RHD		+	+			+		Active right and left auriculitis, myocarditis, endocarditis of mitral and aortic valves with mitral stenosis and insufficiency, pericarditis.
38347	9	4 yr.	RHD								Active myocarditis, endocarditis of mitral, aortic and tricuspid valves with mitral stenosis and aortic insufficiency.
38862	29	Unknown	Pyelonephritis								Active myocarditis; inactive auriculitis.

39619	64	10 yr.; TI,‡ 1 mo.	RHD				Active myocarditis; mitral and aortic stenosis.
39938	44	10 mo.	RHD				Active left auriculitis, myocarditis, endocarditis of mitral valve; mitral and aortic stenosis.
40096	44	More than 5 yr.; TI, 5 mo.	RHD				Active left auriculitis, myocarditis, endocarditis of mitral and aortic valves, pericarditis.
40553	46	2 mo.	RHD				Active myocarditis, endocarditis of mitral and aortic valves.
42364	39	18 yr.; TI, 1 yr.	RHD		+	+	Active right and left auriculitis, myocarditis; inactive endocarditis of mitral, aortic and tricuspid valves with mitral stenosis.
42391	2½	Sore throat 30 days; rh. fever 11 days	RHD	+	+	+	Active left auriculitis, myocarditis, endocarditis of mitral valve.
43055	15	10 days	RHD		+		Active myocarditis, endocarditis of mitral and aortic valves.
A-50-1160	59	20 yr.	RHD		+	+	Active endocarditis of mitral valve.
A-51-1028	8	3 yr.	RHD	+	+	+ +	Active myocarditis, endocarditis of mitral valve, pericarditis.
A-52-1329	4	3 wk.	RHD		+	+	Active left auriculitis, myocarditis, endocarditis of mitral valve.

* A = banded lesion; B = acute inflammatory reaction; C = healed lesion; D = Aschoff bodies.
† RHD = rheumatic heart disease.
‡ TI = terminal illness.

cells about the nutrient vessels were present in the media; these cells appeared to be of connective origin and, where grouped together, formed small nodules that resembled Aschoff nodules of the myocardium. The thrombi were closely adherent to the intima.

Klinge[6] referred in great detail to rheumatic granulomas in the walls of some coronary veins and to "fibrinoid swelling" in others. Similar involvement of extracardiac veins in the neck and thorax was described. Gross, Kugel and Epstein,[7] in reporting the lesions of the coronary arteries in rheumatic fever, mentioned "fibrinoid necrosis" of a coronary vein and in another vein, a verrucous endophlebitis. Baggenstoss[8] in the section on rheumatic disease in Gould's *Pathology of the Heart* stated that rheumatic inflammation of the veins was not uncommon, and one of the illustrations showed a venous mural granulomatous reaction.

Recently there has come to our attention a lesion in the coronary sinuses in cases of rheumatic cardiac disease that is unlike the usual forms of phlebitis and that seemed worthy of further study. No comment can be made concerning gross changes in the sinus wall since the hearts were no longer available for examination.

Material and Methods

The histologic sections from cases of active rheumatic cardiac disease were reviewed to determine in how many instances blocks of tissue had been removed that included the coronary sinus. These were fragments of myocardium that had been taken from the margin of the customary incision to open the left ventricle posteriorly and included the mitral valve and the left atrium. There were found 19 sections from 18 cases that were suitable for this study (Table I). In addition, a block of tissue taken from one of these hearts through the tricuspid valve and right atrium had in it a cross section of the small coronary vein in the right atrioventricular groove. These tissues had been fixed in either Zenker's solution or 10 per cent formalin and were embedded in paraffin.

Additional sections were made from the paraffin-embedded tissues and were stained in various ways: hematoxylin and eosin, phosphotungstic acid-hematoxylin, aniline blue, and Weigert's stain for elastic tissue combined with van Gieson's stain.

Similar sections from 35 individuals of comparable ages were examined; none of these had any clinical or pathologic evidence of rheumatic disease. These served as a control series.

Histologic Alterations in the Sinus and Veins

The histologic alterations were variable; yet they formed a distinctive group. In some instances there were collections of neutrophils in the adventitia of the sinus. This acute reaction was, at times, continued through the media into the intima, resulting in a widening of these layers and especially of the intima (Fig. 1). Nodular collections of large cells with vesicular nuclei and abundant cytoplasm were also to be found in other instances, clustered about fragments of swollen collagen in the adventitia. A prominent, irregular or bar-shaped nucleolus was present

in each nucleus; some of these cells contained two or more nuclei. These cellular accumulations were in every way identical with Aschoff bodies; they occurred singly in the adventitia, at other times were numerous and sometimes lay so closely together that they coalesced (Fig. 2). Occasionally, similar cells seen in the media did not form nodules.

In addition to the acute inflammatory reaction in the intima there was another and very striking intimal alteration that, when present, was an outstanding feature. Clumps of dense, eosin-staining material in the intima formed a prominent band that was bordered by a palisade of perpendicularly arranged large mononuclear cells. Among these mononuclear cells was an occasional cell with two nuclei. Numerous strands of fibrin were present in the eosin-staining areas. The eosinophilic substance and the cellular infiltrate widened the intima and gave to the surface a somewhat wrinkled or corrugated appearance (Figs. 3 and 4). The endothelial cells were swollen over the affected area but were intact. Occasionally, over an area where the intima was widened and inflamed, there was a thin layer of fibrin with the strands parallel to the surface. A thrombus was not found in any instance. The elastica interna was fragmented.

The healing of Aschoff nodules left dense avascular scars in the adventitia. When the reaction in the vessel wall was acute, a granulation tissue sprang up in the adventitia; capillaries traversed the media and penetrated through gaps in the elastic interna into the involved portion of the intima. The intima was thickened by strands of collagen, among which were the new capillaries (Fig. 5). Later, as the lesion became older, the collagen in the intima appeared more compact, and in it a thick-walled vessel with a surrounding infiltration of lymphocytes was occasionally encountered. The intima was thus permanently thickened (Fig. 6).

These lesions affected only sectors of the sinus and extended along the vessel for short distances. Several sectors of the sinus wall might be involved by acute lesions, or there were acute and healing or healed lesions with segments of normal sinus wall between them. In no instance was the sinus affected about its entire circumference at a given level. However, in serial sections, in another sector near by or on the opposite wall of the sinus a new acute focus or a healed lesion could be found. Depositions of calcium were not seen.

The smaller branches of the veins were occasionally acutely inflamed; their walls were infiltrated with neutrophils. Aschoff nodules were found in the adventitia of some without an acute inflammatory reaction. Neither the bandlike lesion nor thrombi were seen in these small venous branches.

In this series of 18 cases, lesions of the coronary sinus, as described, were found in 10. An acute inflammatory reaction of the sinus was found in 8 cases, and in 5 of these there were also Aschoff bodies in the adventitia. Aschoff bodies were present in the adventitia without other reaction in 1 case. In 3 cases there were bands of eosin-staining material in the intima and an acute inflammatory reaction with Aschoff bodies in the adventitia of the sinus. Healed lesions were seen in the sinus in 5 cases, in 4 of these there were also acute lesions.

In none of the control cases were comparable lesions seen.

Discussion

There were close resemblances between the lesions in the coronary sinus and those in the atrial endocardium in rheumatic fever. In the coronary sinus and atrial endocardium, Aschoff nodules were found in the outer layer; also there were acute inflammatory reactions and striking bandlike lesions. The cytologic responses were essentially identical. Whether the involvement of the sinus was an extension of a lesion in the right atrium or an independent lesion could not be determined from this study, as there were no sections that included the terminal portion of the sinus and the right atrium. There was only one atrioventricular section that included the right atrium; in this instance the endocardium was the site of an endocarditis identical with the rheumatic endocarditis in the left atrium. In each of the cases, characteristic rheumatic lesions were present in other parts of the heart. Similar sinus lesions were not found in the control tissues.

These lesions in the coronary sinus apparently developed and healed rapidly or were present before there were any symptoms or clinical evidences of rheumatic cardiac disease. For example, acute lesions were found in the sinus in a child 2½ years of age who died 11 days after the onset of symptoms (#42391). In another child, aged 4 years, only healed and healing lesions of the sinus were present; this child had a clinical history of rheumatic disease of 3 weeks' duration (#A-52-1329). In only 3 of the cases did a coronary sinus have all 3 of the different types of changes described, namely, Aschoff bodies, the acute inflammatory reaction and the bandlike lesion. More often the first two of these lesions were present. As in the rheumatic endocarditis of the left atrium, acute and healed lesions were frequently encountered in a single section of the sinus.

Involvement of the entire circumference of the sinus was not observed; the damage was limited to sectors of the sinus wall, though in serial sections several sectors might be involved at different levels. This localized involvement was noted previously by Shaw.[3] The absence of

thrombi associated with phlebitis of the sinuses seemed remarkable in view of the extensive damage to these vessels; this has also been mentioned by Shaw. Failure of thrombus formation is likewise a feature of rheumatic arteritis,[9] as it is in auriculitis[10,11] and in involvement of the auricular appendage.[12]

The phlebitis of the coronary sinus should produce a grossly visible lesion; at least the thickening of the intima seemed to be sufficiently marked for it to be noted. One may only speculate about possible rheumatic lesions in the veins in other parts of the body. Judging by the few recent reports concerning rheumatic phlebitis, careful studies of veins do not appear to have been made, even though rheumatic arteritis and aortitis have been recognized for many years. Perhaps this is due to the absence of thrombosis, since this would be most likely to attract attention to a venous lesion. It would not be surprising to discover that the veins are more often damaged in rheumatic fever than is presently believed; indeed, one might anticipate this to be true in view of the involvement of the arterial portions of the vascular system.

Summary

There was found in the coronary sinus of the heart a distinctive lesion in rheumatic fever that was different from any of the usual forms of phlebitis. The lesion involved only sectors of the sinus wall and in its structure closely resembled rheumatic endocarditis of the left atrium. The phlebitis apparently developed and healed rapidly or might exist before there was clinical evidence of rheumatic cardiac disease. The presence of acute and healed lesions in the same sinus would indicate that the vessel was being damaged repeatedly. There was an absence of thrombosis with this lesion.

References

1. Sladen, F. J., and Winternitz, M. C. Venous thrombosis during myocardial insufficiency. *Johns Hopkins Hosp. Rep.*, 1916, **17**, 511–550.
2. Pappenheimer, A. M., and Von Glahn, W. C. Studies in the pathology of rheumatic fever. Two cases presenting unusual cardiovascular lesions. *Am. J. Path.*, 1927, **3**, 583–594.
3. Shaw, A. F. B. Topography and pathogenesis of lesions in rheumatic fever. *Arch. Dis. Childhood*, 1929, **4**, 155–164.
4. Gräff, S. Primaerinfekt und invasionsstelle beim rheumatismus infectiosus specificus. *Deutsch. med. Wchnschr.*, 1930, **56**, 603–606.
5. Perry, C. B.; Davis, O. C. M., and Schlessinger, B. Thrombo-phlebitis in acute rheumatism. With clinical case notes. *Lancet*, 1933, **2**, 966–969.
6. Klinge, F. Der Rheumatismus; pathologisch-anatomische und experimentell-pathologische Tatsachen und ihre Austwertung für das ärztliche Rheumaproblem. *Ergebn. allg. Path. u. path. Anat.*, 1933, **27**, 1–351.

7. GROSS, L.; KUGEL, M. A., and EPSTEIN, E. Z. Lesions of the coronary arteries and their branches in rheumatic fever. *Am. J. Path.*, 1935, 11, 253–280.

8. BAGGENSTOSS, A. H. Rheumatic Disease of the Heart. In: Pathology of the Heart. GOULD, S. E. (ed.) Charles C Thomas, Springfield, Ill., 1953, pp. 638–692.

9. VON GLAHN, W. C., and PAPPENHEIMER, A. M. Specific lesions of peripheral blood vessels in rheumatism. *Am. J. Path.*, 1926, 2, 235–250.

10. MACCALLUM, W. G. Rheumatism: the Harrington lecture. *J.A.M.A.*, 1925, 84, 1545–1551.

11. VON GLAHN, W. C. Auricular endocarditis of rheumatic origin. *Am. J. Path.*, 1926, 2, 1–14.

12. KUSCHNER, M., and LEVIEFF, L. Correlation between active rheumatic lesions in the left auricular appendage and elsewhere in the heart. *Am. J. M. Sc.*, 1953, 226, 290–295.

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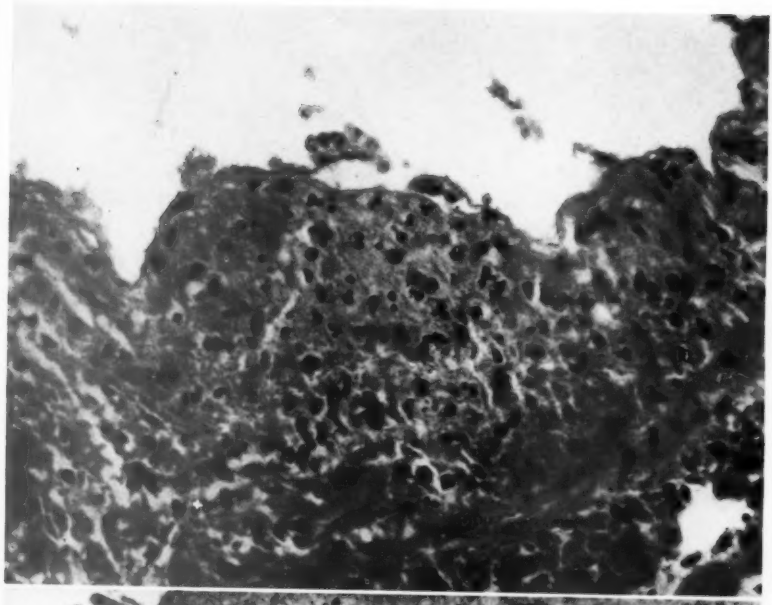
LEGENDS FOR FIGURES

All illustrations were prepared from sections stained with hematoxylin and eosin.

FIG. 1. Acute inflammatory reaction in the intima of a coronary sinus. The intima is widened. There is no thrombus over the area. × 300.

FIG. 2. Section through a vein in the right atrioventricular groove, showing two Aschoff nodules in the adventitia. One of the Aschoff cells is multinucleated. × 300.



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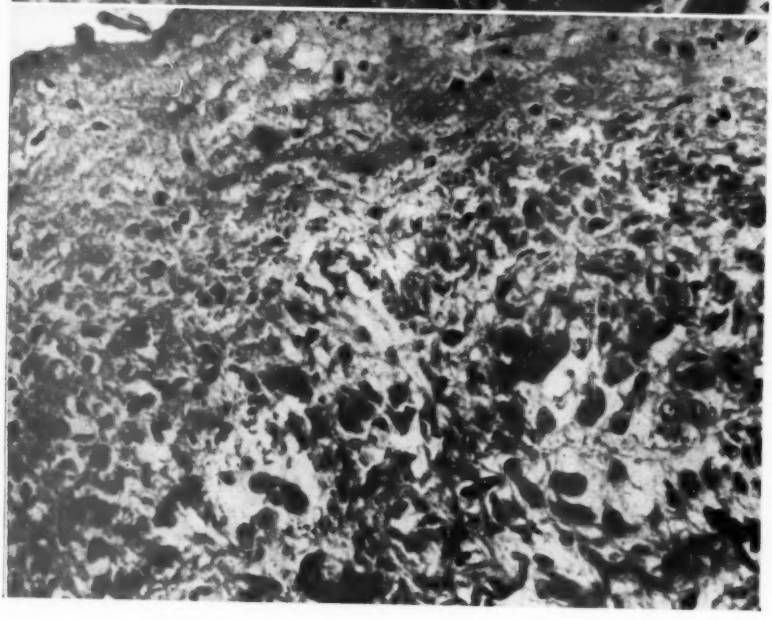

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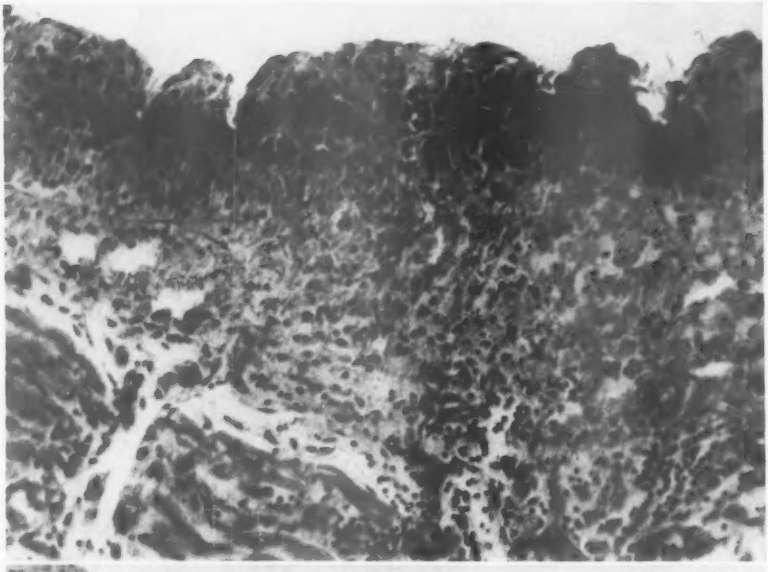

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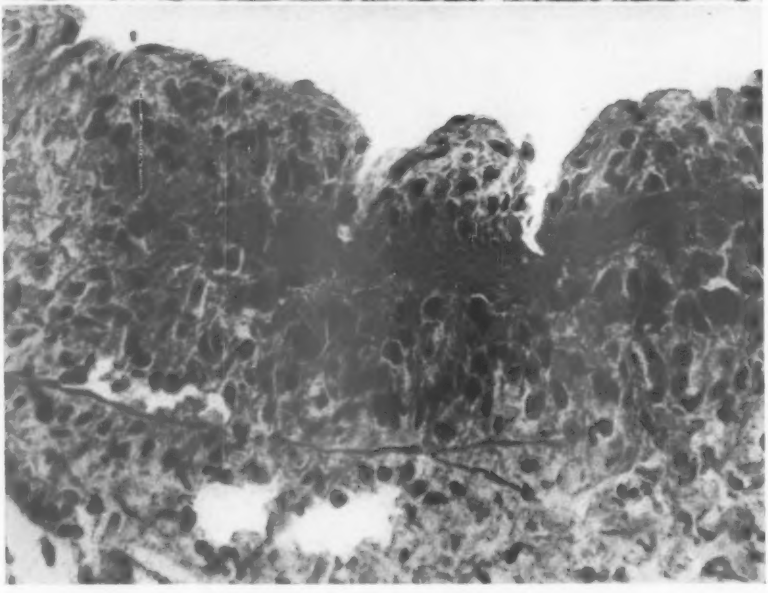
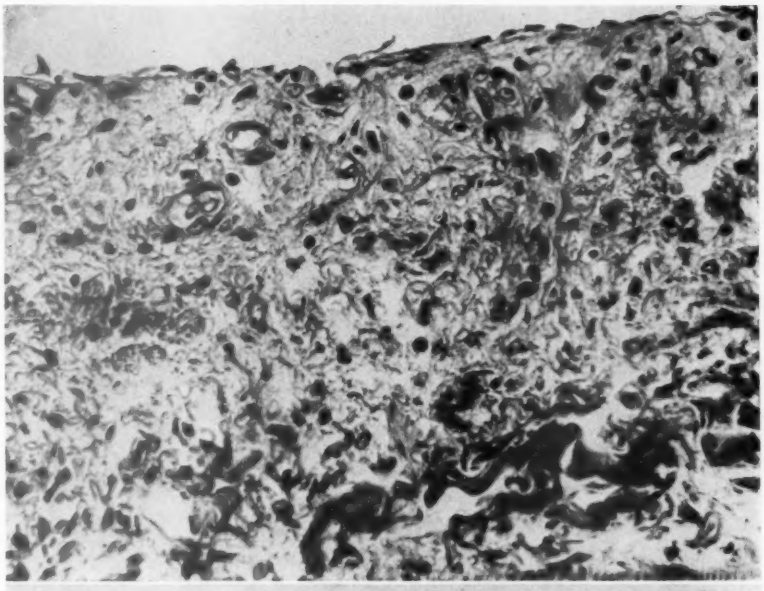


FIG. 3. Coronary sinus. The intima is greatly widened by a broad band of compact, eosinophilic substance bordered by perpendicularly arranged large mononuclear cells. The endothelium is intact. There is no thrombosis. In the adventitia is an acute inflammatory reaction; neutrophils also infiltrate the media. × 150.

FIG. 4. Higher magnification of a portion of the lesion shown in Figure 3. The internal elastic lamella is split. × 300.



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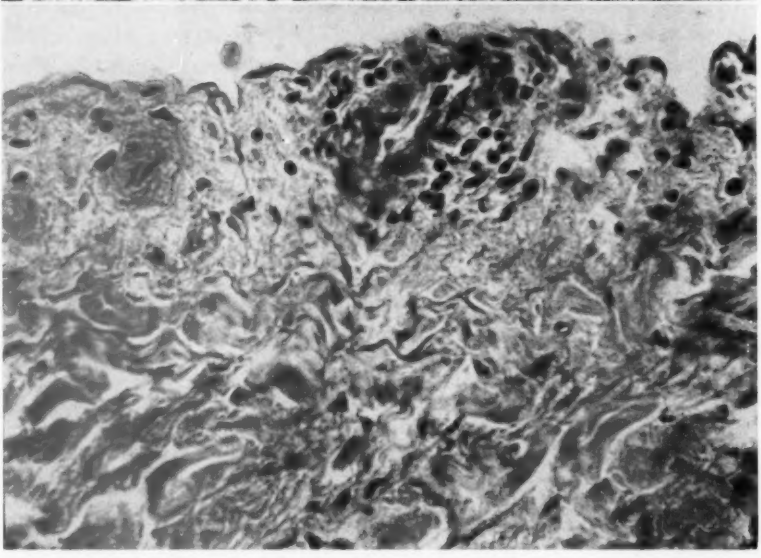

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FIG. 5. Coronary sinus; healed lesion. The intima is widened by strands of collagen, with large capillaries. × 300.

FIG. 6. Coronary sinus; healed lesion. The intima is thickened by compact collagen in which a thick-walled vessel is surrounded by lymphocytes. This healed lesion is in the sinus wall directly opposite the acute lesion shown in Figures 3 and 4. × 300.